

بسم الله الرحمن الرحيم

دراسات على بكتيريا *Staphylococcus aureus* المعزولة من بعض العاملين في مجال تداول الأطعمة وقابليتها لإنتاج السموم المعوية في بعض الأكلات السعودية المطبوخة

للطالب/انس بن سراج عبد الرحمن دبلول

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رسالة مقدمة للحصول على درجة الماجستير

في

ميكروبيولوجيا الأغذية و التسممات الغذائية

المشرفون

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بسم الله الرحمن الرحيم

وزارة التعليم العالي
جامعة أم القرى
كلية العلوم التطبيقية

نموذج رقم (٨)

إجازة أطروحة علمية في صيغتها النهائية بعد إجراء التعديلات

الاسم (رباعي) انيس بن سراج عبدالرحمن دبلول كلية : العلوم التطبيقية قسم : الأحياء - ميكروبيولوجيا .

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عنوان الأطروحة : ((دراسات على بكتيريا S. aureus المعزولة من بعض العاملين في مجال تداول الأطعمة وما ينتجه من السموم المعوية في بعض الأكلات السعودية المطبوخة

الحمد لله رب العالمين والصلاة والسلام على أشرف الأنبياء والمرسلين وعلى آله وصحبه أجمعين وبعد :

فبناءً على توصية اللجنة المكونة لمناقشة الأطروحة المذكورة أعلاه والتي تمت مناقشتها بتاريخ ٤/٦/١٤٢٤هـ بقبولها بعد إجراء التعديلات المطلوبة، وحيث قد تم عمل اللازم ؛ فإن اللجنة توصي بإجازتها في صيغتها النهائية المرفقة للدرجة العلمية المذكورة أعلاه ...

والله الموفق ...

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Studies on Isolates of *Staphylococcus aureus* From Food Handlers and Their Ability to Produce Enterotoxins in Some Saudi Cooked Food.

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THESIS

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Dedication

To my mother Fozeyh for here patience, support, invocation and devotions
To my Father's Serag who made it all possible even after his death
To Rosa and my flowers Alaa, Serag, Loui and Hazm for their love
To my brother and sisters for their invocation

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Table of contents Page No.

MATERIALS AND METHODS

Results and Discussion

Abstract

The present investigation is concerned with isolation, purification, and identification of *Staphylococcus aureus* isolated from some food handlers whom applied to work in hospital-located kitchens in Makkah during high seasons of Hajj. Out of 129 *Staphylococcus aureus* isolates from 1516 clinical specimens from food handlers of different nationalities; 35% produced enterotoxins A, B, C and D singly or in pairs, when such enterotoxins were evaluated by Reversed Passive Latex Agglutination test (RPLA).

Enterotoxins C and A, elaborated by 15.5% and 12.4%, isolates respectively, which showed the highest percentage. They were mostly isolated from nasal swabs than throat swabs. All isolates were resistant to Penicillin G. On the other hand, they were sensitive to Clindamycin, Oxacillin and Gentamicin when tested by Kirby-Bauer method.

So, two isolates each forming either SEA or SEC were chosen, and were subjected to further studies, i.e., growth curve in selected medium and in cooked rice. The effect of the holding temperature of rice on the production of SEA and SEC was evaluated.

Many conclusions could be drawn but the most important one is the necessity to reactivate the role of the health certification, so it should cover the training and educational part beside the medical examinations.

Abbreviation

a_W	:	Water activity
cAMP	:	Cyclic Adenosine Monophosophate
CDC	:	Center for Disease Control, Atlanta USA
CIOMS	:	The Council for International Organizations of Medical Sciences
CPE	:	*C. perfringens* Enterotoxin
D	:	Dalton
DNase	:	Deoxyribonuclease
ED_{50}	:	The 50 % Emetic dose
ELISA	:	Enzyme-Linked Immuno-Sorbent Assay
US FDA	:	US Federal and Drug Administration
I^{125}	:	Radioactive Iodine
IEA	:	Immunoenzymatic Assay
Mab	:	Monoclonal antibody
MIC	:	Minimal Inhibitory Concentration
MLC	:	Minimal Lethal Concentration
MRSA	:	Methicillin-Resistant *S. aureus*.
MSSA	:	Methicillin-Susceptible *S. aureus*.
PCR	:	Polymerase Chain Reaction
PAGE	:	Poly Acrylamide Gel Electrophoresis
PCR	:	Polymerase Chain Reaction
PIGE	:	Pulsed-Inversion Gel Electrophoresis of total DNA
PHA	:	Passive Hemagglutination Assay
PFGE	:	Pulsed-Field Gel Electrophoresis
RIA	:	Radio-Immuno -assay test
RPHA	:	Reversed Passive Hemagglutination Assay
RPLA	:	Reversed Passive Latex Agglutination test
SaG	:	An enzyme produced by the *S.aureus* endo-acetylglucosaminidase
SEA	:	Staphylococcal EnterotoxinA
SEB	:	Staphylococcal EnterotoxinB
SEC	:	Staphylococcal EnterotoxinC
SED	:	Staphylococcal EnterotoxinD
SEE	:	Staphylococcal EnterotoxinE
SEH	:	Staphylococcal EnterotoxinH
SEG	:	Staphylococcal EnterotoxinG
SEI	:	Staphylococcal EnterotoxinI
SEs	:	Staphylococcal Enterotoxins
SRD	:	Single Radial immunodiffusion
SSSS	:	Staphylococcal Scalded Skin Syndrome
TSS	:	Toxic Shock Syndrome
TC	:	Total count
Tnase	:	Thermonuclease TSST-1 :Enterotoxin F
WHO	:	World Health organization

List of Tables

List of illustrations

Introduction

Many inhabitants in Saudi Arabia nowadays depend on prepared cooked foods presented in many restaurants and pantries especially in big cities as Holy Makkah, Madina, and Jeddah, all year around. Of special importance is during Hajj, and Omra seasons.

Hajj is Pilgrimage season when more than 2 million people (in specific time throughout the year) gather in Makkah and Holy areas of Arafat, Mena, and Mozdalifa to perform religious rituals of worship. On the other hand, Omra seasons throughout the year focused mainly on Makkah where visitors worship in the Holy Mosque, in addition to large number of visitors to Madina.

In ordinary times, food handlers are subjected to medical examination before assignment to work in food stations. However, during high seasons of work, i.e., Hajj and Omra, those establishments employ temporary workers even from other countries; mostly lacking proper training in food handling operations, mass feeding, and sanitary practices. This situation may encourage contamination with microorganisms both causing food spoilage and food intoxication. Of most widespread intoxication, which depends largely on sanitary practices is staphylococcal food poisoning.

Object of Investigation

The present investigation is concerned with isolation, purification, and identification of *Staphylococcus aureus* isolates from some food handlers who applied to work in Makkah during high seasons. Swabs will be obtained directly from throat, nose, nails, wounds, and stool samples. The isolates will be characterize and tested for the ability to produce enterotoxins in culture media, and in some Saudi traditional cooked foods such as Mandy and Bokhary rice. Mandy and Bokhary rice dishes are very popular, staple food, and are served in public kitchens spreading everywhere in the Kingdom.

Review of Literature

1.1 Food borne diseases

ICD-9005; ICD-10A05

1.1.1 Definition

Foodborne Diseases are defined as conditions of distress following the ingestion of contaminated food and or drink (Ayres *et al.*, 1980). As they are widespread and cause public health problems and concern, theWorld Health organization WHO's International Committee for Classification of Diseases in its 9th Revision identified Foodborne intoxications and Foodborne infection, as terms applied to illnesses acquired by consumption of contaminated food (Benenson and chin, 1995).

On the other hand, to avoid confusion based on nosology in different languages, each disease is identified by numbers and English names, which were recommended by the Council for International Organizations of Medical Sciences (CIOMS), and the WHO in the International Nomenclature of Diseases. Food borne diseases designated as "ICD-9005; ICD-10A05". However, the simplest definition for food borne diseases is diseases transmitted by foods (WHO, 2000a).

Food borne diseases are frequently classified on the basis of the type of agent that is responsible for illnesses such as bacterial, viral, parasitic, fungal, poisonous plants, toxic animals, and poisonous chemicals. Bryan (1976) classified food borne diseases into food poisonings and food infections. In 1984, Frazier and Westhoff reported that food poisonings could be the result of either

chemical poisoning or the ingestion of a toxicant (intoxication). The later could be caused by certain plants or animal tissues, and metabolic products (toxins) formed and or excreted by microorganisms, i.e., bacteria, fungi, algae, during their growth and multiplication in food (Heritage *et al.*, 1999).

The entrance of pathogenic microorganisms into the body and the reaction of body tissues to their presence or to the toxins they generate within the body cause food infections (Bryan, 1976).

1.1.2 Estimation of Food borne Diseases

There are more than 200 well-known food borne diseases reported by Mead *et al.*, (1999). The global incidence of foodborne diseases is difficult to estimate, because of many reasons including:

1. The absence of good surveillance systems in most of the Third World countries.
2. Lack of reliable information on the magnitude of the problem.
3. Milder cases are often not detected or reported.
4. Some foodborne diseases are caused by pathogens that have not yet been identified (WHO, 1997a). But it has been reported that in 1998 alone 2.2 million people died from diarrheal diseases (Mead *et al.*, 1999). Most of these cases can be attributed to contamination of food and drinking water. However, it is estimated that the reported cases represent less than 10%, of the real incidence (Motarjemi and Kaferstein, 1997).

1.1.3 Bacterial food poisoning

Of all the causes of food poisoning, the most common is bacterial food poisoning (Heritage *et al.,* 1999). Two groups of food poisoning bacteria are recognized. The first is known as toxin-producing bacteria, and the second as infecting bacteria (Prescott *et al.*, 1990).

The toxin-producing bacteria include any bacteria that can produce toxins. They are divided into two sub-groups, the first, includes the bacteria that usually liberate the toxin in the food prior to ingestion, i.e., *S. aureus* and *Clostridium botulinum*. On the contrary, the second sub-group, produce toxin after being ingested by the victim, i.e., *Vibrio cholerae* and *C. perfringens* (Lederberg, 2000). The produced toxin is responsible for eliciting the clinical manifestation of the disease. It may not be necessary to ingest viable bacteria from the first group to suffer from an intoxication type (Heritage *et al.,* 1999).

Infecting food bacteria are those primarily invade the intestinal epithelial cells after entering the body through ingestion of contaminated foods, i.e., *Salmonella spp.* and *Campylobacter spp.* (Frazier and Westhoff, 1984).

WHO estimated that more than 5.2 million food illnesses in U.S.A are due to bacteria each year, which represent 13% of the total cases of the food borne diseases that are caused by known pathogens (Mead *et al.*, 1999).

1.1.3.1 Factors contributing to Bacterial food poisoning

According to the WHO's issue "Statistics Quarterly on Outbreak" (WHO, 1997b); in spite of improvements in methods of food preparation, and education of those responsible for the provision of food, which is expected to reduce the incidence of food poisoning. It can be seen that rather than narrow; food borne illnesses actually appears to be increasing (WHO, 2000b).

To accomplish this; it is essential to know all about the science of food hygiene, which aims to produce food that is safe to the consumer and of good keeping quality. It covers a very wide field, and includes: the rearing, feeding, marketing, and the sanitation procedures designed to prevent bacteria of human origin from reaching the foodstuff (Jaad, 1997). The largest proportion of the outbreak's incidents occurred from food prepared in restaurants, hotels, clubs, hospitals, schools, etc. (Hobbs and Roberts, 1990).

Various characteristics of the food itself may allow or inhibit bacterial multiplication. These include factors such as pH, water activity (a_w), and level of organisms competing with or inhibiting the growth of potential pathogens (Ayres *et al.*, 1980). On the other hand, certain characteristics of food poisoning bacteria contribute to the incidents, i.e., production of heat-resistant spores, ability to grow at relatively high or low temperature, and tolerance of high salt or sugar levels (Frazier and Westhoff, 1984).

However, for all types of food poisoning; the factors, which are recorded as most commonly contributing to outbreak as reported by Hobbs and Roberts, (1990) include:

1) The use of contaminated processed food.
2) Preparation of food more than half a day in advance of needs.
3) Undercooking and raw food consumption.
4) Storage at ambient temperature or improper warm holding.
5) Inadequate cooling and inadequate reheating.
6) Cross-contamination from raw to cooked food.
7) Use of the left over food.

1.1.3.2 Major toxin-producing bacteria

- ***Bacillus cereus.***

ICD-9 5005.8;ICD-10 A05.4

B. cereus is a Gram-positive, spore-forming aerobe that is present in soil, vegetation, water, and dust (Jawetz *et al.*, 1989). Two toxins are responsible for clinical illnesses: the first is an emetic heat-stable toxin that causes vomiting, which resembles staphylococcal food poisoning (a short incubation period emetic syndrome). Boiled rice stored at room temperature has often been implicated in these outbreaks. The second is a heat-labile enterotoxin that is associated with diarrhea, and clinically resembles *Clostridium perfringens* food poisoning. Implicated foods include poultry, cooked meats, mashed potatoes, various soups, and desserts (a longer incubation period cause diarrheal syndrome) (Lederberg, 2000).

- ***Vibrio cholerae.***

ICD-9 001; ICD-10 A00

The organism was first described and named by Pacini in 1854; thirty-two years latter Koch isolated the same organism, and renamed it 'Kommabacillus' because of the characteristic curved or comma-shaped appearance (Lederberg, 2000). Currently, there are over thirty Vibrio species. Vibrios are gram-negative facultative aerobes, and thiosulfate citrate bile salts agar is frequently used for isolation. Two main types of *Vibrio cholerae* strains have been identified: *Vibrio cholerae* O1, and *Vibrio cholerae* O139 or "non-O1"(Albert, 1994). *Vibrio cholerae* O1 is divided into two biotypes: classical and El-Tor, and can be further separated into one of three serogroups: Inaba, Ogawa, and Hikojima. *V. cholerae* is spread via contaminated water and food (Jawetz *et al.*, 1989). The organism's principal virulence factor is the production of cholera toxin that is an activator of cyclic adenosine monophosophate (cAMP) in intestinal epithelial cells. This results in watery diarrhea, and if untreated, may cause dehydration leading to death. A number of other members of the Vibrio species have been associated with food borne diseases, including *V. parahemolyticus,* ICD-9005.4; ICD-10A05.3, and *V. vulnificus,* ICD-9005.8; ICD-10A05.8 (Lederberg, 2000).

- ***Clostridium perfringens.***

ICD-9 005.2; ICD-10A05.2

C. perfringens is an anaerobic, spore-forming, gram-positive rod that is frequently found in excreta from humans and animals. It is also found in raw meats, and poultry. There are five types of *C. perfringens* (A to E); Jawetz *et al.*, (1989). Of the various types of C. *perfringens* type A is the one predominantly associated with the consumption of cooked meat or poultry, which has been allowed to remain between 15 - 60°C for more than 2 hrs, and this results in a noninflammatory diarrhea (Lederberg, 2000). *C. perfringens* enterotoxin (CPE) is a heat-labile 35000 Dalton (D) protein encoded by the *cpe* gene. It has a complex mechanism of actions and appears to insert itself into the host cell membrane to form a protein complex. This result in membrane permeability changes, and losing of intracellular potassium (Koneman *et al.*, 1997).

- ***Escherichia coli.***

ICD-9 008.0;ICD-10 A04.0-A04.4

E. coli is a short gram-negative rod that may form chain. Typically, it produces positive tests for indole, lysine decarboxylase, mannitol fermentation, and produces gas from lactose (Jawetz, 1989). The majority of *E. coli* species in the gastrointestinal tract are harmless unless displaced to other part of the body, i.e., urinary tract. The pathogenic *E. coli* species are

divided into six groups according to their actions in the body as reported by Benenson and chin (1995), and Lederberg, (2000):

1- Enteropathogenic (EPEC), ICD-9 008.0;ICD-10 A04.0.
2- Enterotoxigenic (ETEC), ICD-9 008.0;ICD-10 A04.1.
3- Enteroinvasive (EIEC), ICD-9 008.0; ICD-10 A04.2.
4- Enterohaemoorhagic (EHEC) ICD-9 008.0;ICD-10 A04.3.
5- Enteroadherent (EAEC), ICD-9 008.0;ICD-10 A04.4.
6- The relatively newcomers Shiga toxin-producing *E. coli.*

1.2 *Staphylococcus*

1.2.1 Historical Introduction

More than hundred years have elapsed since cocci were first observed in diseased tissues and in pus obtained from human abscesses. These were called "*Micrococci*" by Von Recklinghausen (1871), *Microsporon septicum* by Klebs in 1872 and "*monads*" by Hueter in 1872 (Ogston, 1882). In 1880, Ogston and at the same time, Pasteur concluded that a cluster-forming coccus was the cause of certain pyogenic abscess in man. That organism was named "*Staphylococcus*" (Ogston, 1882).

The *genus* Staphylococcus was derived from the Greek word *staphule* (a bunch of grapes) and *kokkus* (a grain or berry) (Hine, 1998). Afterwards, the name aureus and albus were used to distinguish different species depending on the color of colonies (gold, and white, respectively) on the blood agar medium (Heritage *et al.*, 1999).

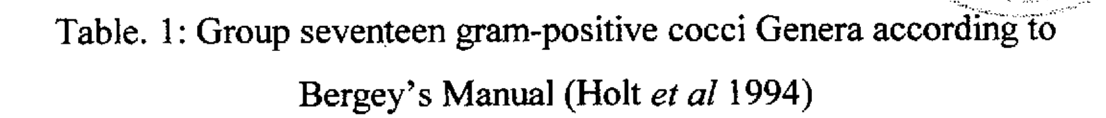


Table. 1: Group seventeen gram-positive cocci Genera according to Bergey's Manual (Holt *et al* 1994)

Genus **Micrococcus**	***Genus*** **planococcus**	***Genus*** **Ruminococcus**
Genus **Enterococcus**	***Genus*** **Coprococcus**	***Genus*** **Deinobacter**
Genus **Lactococcus**	***Genus*** **Salinicoccus**	***Genus*** **Deinococcus**
Genus **Marinococcus**	***Genus*** **Leuconostoc**	***Genus*** **Saccharococcus**
Genus **Lactococcus**	***Genus*** **Pediococcus**	***Genus*** **Staphylococcus**
Genus **Melisscoccus**	***Genus*** **Sarcina**	***Genus*** **Trichococcus**
Genus **Gemealla**	***Genus*** **Vagococcus**	***Genus*** **Peptostreptococcus,**
Genus **Streptococcus**	***Genus*** **Aerococcus**	***Genus*** **Stomatococcus**

1.2.2 Classification

The *genus* Staphylococcus listed within the second Major category (Gram- positive eubacteria that have cell wall) in-group seventeen (gram-positive cocci) according to Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994), this group consists of twenty-four genera as shown in Table (1)

The *genus* Staphylococcus is currently composed of thirty-three species (Holt *et al.*, 1994); seventeen species were found in man and primates, twelve species were found in other animals (Biberstein *et al.*, 1984), and four species mostly environmental (Koneman *et al*, 1997). Only three of them are coagulase-positive (Roberson *et al.*, 1992). Humans may become colonized or infected by members of this Genus due to frequent contact with animals. Among the Staphylococci frequently involved in human infections are *S. aureus* (coagulase-positive), *S. saprophyticus*, and *S.epidermidis* (coagulase-negative) (Degener *et al.*, 1994).

1.2.3 Morphology and Characteristics

1.2.3.1 Cell morphology and physiology

Staphylococci are spherical, 0.5-1.5 μm in diameter, occurring singly, in pairs, and in irregular clusters because they divide in more than one plane (Prescott *et al.*, 1990). Young cocci are strongly gram positive; on aging, however, many cells become gram variable. They are nonmotile and do not form spores. The staphylococci are facultative anaerobes, and Chemo-organo-trophic

in both respiratory and fermentative metabolic test (Holt *et al.*, 1994). They are usually catalase positive, and oxidase negative. However, nitrate is often reduced to nitrite (Koneman *et al.*, 1997).

1.2.3.2 Cultures

Staphylococci grow readily on most bacteriologic media (Lachica, 1984). On the solid media colonies are 1.5-2.5mm round, smooth, raised, and glistening. Colonies are usually opaque producing pigments that vary from white, cream, yellow to orange (Holt *et al.*, 1994).

The optimum temperature for the growth is 30-37 °C, but pigment formation is best between 20-25°C. They are relatively resistant to heat up to 50°C for 30 minutes Tuncan and Martin, (1990). It can tolerate up to 10% sodium chloride (Chapman, 1945a). They are susceptible to lysis by lysostaphin but not by lysozyme (Garcia *et al.*, 1980).

1.2.3.3 Growth media

It is common for staphylococci to be present in mixed cultures with a variety of other organisms in carriers, feces, air, wounds, food, and in various surfaces. In addition, the total number of organisms present may be very large, i.e., 10^8 to 10^{10} per gram in feces (Finegold and Sweeney, 1961). The staphylococci may form a relatively small percentage of the total bacterial population. Furthermore, when staphylococci are present in mixed culture with certain Gram-negative bacilli, the later could suppress the

staphylococci, (Noleto, *et al.*, 1987) so that the recovery is difficult except by the use of selective media (Devriese and Hajek, 1980). Several media have been developed, which assist in rapid identification such as Mannitol salt agar (Finegold, and Sweeney, 1961), *Staphylococcus* medium no. 110, (Niskanen and Aalto, 1978), and Baird Parker medium (Lachica, 1984).

1.2.4 Staphylococcal Diseases

These organisms are the most bacteria that cause diseases in human ranging from a single pustule to sepsis and death. They are normally found in: the upper respiratory tract, skin, intestine, human vagina (Prescott *et al.*, 1990) and in animal (Biberstein *et al.*, 1984). Virulence varies from one strain to another but the most important pathogen to humans is coagulase positive *Staphylococcus aureus* (Roder *et al.*, 1995). However, coagulase negative strains are increasingly more important in bloodstream infection (intravascular catheters), urinary tract infections of women, and in hospital-acquired infections (Benenson and Chin, 1995).

The WHO's International Classification of Diseases (ICD) (9^{th} revision) divided staphylococcal diseases into four groups each group has a specific code because staphylococcal diseases have distinctly clinical and epidemiologic patterns in community, in newborns, and among hospitalized patients (Benenson and Chin, 1995):

i. Staphylococcal diseases in the community

ICD-9680, 041.1; ICD-10l02

Boils, carbuncles, furuncles, abscesses, impetigo cellulites, sepsis, pneumonia, arthritis, endocarditis, and osteomyelitis

ii. Staphylococcal diseases in hospital nurseries

ICD-9684, 041.1; ICD-10L00

Staphylococcal Scalded Skin Syndrome (SSSS), abscess of the breast

iii. Staphylococcal diseases in hospital and surgical wards

ICD-9998, 5; ICD-10T81.4

iv. Toxic Shock Syndrome (TSS) ICD-9 785.5; ICD10 A48.3

1.3 Staphylococcal food intoxication

ICD-9005.0;ICD10A.5.0

1.3.1 Introduction

Staphylococcal food poisoning (enterotoxicosis) is classified as a disease of moderate severity (Mossel and Netten, 1990). The relative incidence in various countries is varied. In France, it has been recorded since the beginning of the 19th century (Oliver, 1830). Between 1977 and 1981 in USA Staphylococcal food intoxication was the second most common cause of reported foodborne illness (Holmberg and Blake, 1984). The Center for Disease Control, Atlanta USA (CDC) estimated that staphylococcal food intoxication approximately causes 185060 cases, 1753 hospitalizations, and 2 deaths each year in USA (Mead *et al.*, 1999). In UK, it is possible that Staphylococcal food intoxication is the most common cause of food poisoning (Heritage *et al.*, 1999).

In Canada, it is responsible for one-third of reported food poisoning incidents (Peterkin and Sharpe, 1984). Between 1986 and 1995 *S.aureus* was the next most common food borne pathogen in Taiwan (Pan *et al.*, 1997). Previous unpublished data has shown that staphylococcal food intoxication is the most common food poisoning in Makkah between 1996-1998.

1.3.2 Symptoms

The symptoms of staphylococcal food intoxication depend on the dosage of the enterotoxin consumed, and ranges from being barely noticeable to deaths, which is rare. It is not a reportable disease (Heritage *et al,* 1999). The symptoms in general are vomiting, nausea, cramps, prostration, diarrhea, sometime subnormal temperature and lowered blood pressure (Prescott *et al.*, 1990). The duration of the illness is from one to two days, and in severe cases it may require hospitalization.

1.3.3 Enterotoxigenicity of staphylococci

Many previous unsuccessful attempts have been published to relate enterotoxigenicity to some other properties as: thermostable nuclease, deoxyribonuclease, and coagulase (Casman and Bennett 1965) and (Brandish and Willis, 1970). In general, there is a good correlation with coagulase, but some isolates that are coagulase-negative have been reported to produce enterotoxins (Udo *et al.*, 1999). Practically, all food poisoning outbreaks are due to coagulase positive staphylococci. Data on the

enterotoxigenicity of *S. aureus* strains isolated from humans and foods indicated that more than 50 % are enterotoxigenic (Jawetz *et al.*, 1989).

1.3.4 Food involved and source of contamination

The range of foods commonly contaminated with *S. aureus* are broad including; cooked shrimp (Silverman *et al.*, 1961) raw and cooked meats (Genigeorgis *et al.*, 1971a and Kotzekidou, 1992), egg products, natural and synthetic creams, macaroni, pies, high-salt foods, canned mushrooms (Brunner and Wong, 1992), milk (Harvey and Gilmour, 1985, Hewedy *et al.*, 1990a and Iannelli *et al.*, 1998), and milk products (Bautista *et al.*, 1988), frozen precooked foods (Raj and Liston, 1961), dry cured Iberian ham (Rodriguez *et al.*, 1996), and cheese (Gaya *et al.*, 1988). However, with the exception of dairy products, where the staphylococci may originate from a mastitis infection of animals (Hewedy *et al.*, 1990b and Forsman *et al.*, 1997),

Virtually all incriminated foods have been touched or handled by humans (Lim, 1998). On the other hand, the competing bacteria of most heated foods (any type of heat treatment) that interfere with the growth of the staphylococci, will be lost, which will allow the staphylococcus activity especially if the heated food is re-contaminated from encountered source (Noleto and Bergdoll, 1980).

1.3.5 Investigations

The Public Health Laboratory Service Communicable Disease Surveillance Center (UK) described some definitions, which are very important during food poisoning investigations in the following terms, (Wieneke *et al.*, 1993):

- A case is a person with symptoms from whom the relevant organism has been isolated or who has been affected in an outbreak of food poisoning
- An outbreak is defined when there are two or more related cases of food poisoning, and classified as family or general outbreak
- Sporadic cases occur when an affected patient has had no known association with another person and infected with the same organism.

From the epidemiological viewpoint; the main purposes of the investigations of an outbreak are to find out the responsible source of contamination, and to determine the organism that was involved, and to prevent a repetition of the same set of conditions (Benenson and Chin, 1995).

Investigations consist of gathering information, collection of food samples, collection of specimens from human sources and the laboratory tests. The results of these investigations can lead to incriminated food and the allocation and elimination of the ultimate source of responsible organisms (Hobbs and Roberts, 1990).

1.4 Characteristics leading to *S. aureus* identification

1.4.1 Introduction

All *S. aureus* isolated from humans or animals, when grown *in vivo* or *in vitro*, produce several extracellular enzymes and some other active factors that contribute to their virulence (Blobel *et al.*, 1959). These enzymes play an important role in the identification tests, which include: Catalase, Coagulase, Deoxyribonuclease (DNase) or Thermonuclease (TNase), Hemolysins, and Enterotoxins (Jawetz *et al.*, 1989). On the other hand, there are some conventional tests including: Gram staining, Motility, Oxidase, and Mannitol fermentation, which are essential for identifying *S. aureus.*

1.4.2 The Catalase

Catalase is one of the several enzymes produced by *S.aureus* (Holt *et al.*, 1994). It prevents the accumulation of hydrogen peroxide (H_2O_2), by it's conversion to water and nascent oxygen. The molecular weight is 232,000 D, and produced in small amounts during the logarithmic growth phase, and in larger quantities during the stationary phase (Jensen and Hyde, 1963). It is attached to the intracellular membrane in an inactive form, and when released from the membrane; becomes activated (Martin *et al.*, 1976). Catalase is very sensitive to heat, and loses its activity at 35°C, and at pH near 7. The *genus* Staphylococcus is differentiated

from the *genus* Streptococcus by the catalase test (Holt *et al.*, 1994).

1.4.3 The Coagulase

The correlation between the pathogenicity of certain staphylococci and their ability to produce coagulase has led to extensive studies of the clotting enzymes (Blobel *et al.*, 1959). Most strains of *S. aureus* produce Staphylocoagulase (free coagulase) and Bound coagulase (clumping factor), which cause plasma to clot by converting fibrinogen to fibrin (Luijendijk, 1996).

I - Staphylocoagulase (Free coagulase)

In 1903, Loeb observed that *S. aureus* caused coagulation of goose plasma *in vitro.* He indicated that an enzyme was formed in the culture, which converts fibrinogen to fibrin by activating a coagulase reacting factor present in plasma. Staphylocoagulase is an extracellular single-chain protein with a molecular weight of 61000 D (Engels *et al.*, 1978). It is thermolabile and has isoelectric point of pH 4.53. It can be detected by using the tube test. It is produced as early as the log phase and continues throughout the logarithmic phase (Duthie, 1954). The limitations of this method are: the type of plasma used, the length of incubation, the degree of clot formation, and false-positive and false-negative results have been indicated as sources of error (Baker *et al.*, 1985). But it is usually recommended to perform the tube test on all negative slide coagulase test strains (Cheesbrough, 1985).

Table (2): Some of the coagulase test commercial kits

Kit	Manufacturer
StaphAurex	Murex, Norcross, GA
Staphaurex Plus	Murex Diagnostics Limited
Pastorex Staphplus	Sanofi, Marnes-La-Coquette, France
Slidex Staph	BioMerieux-Vitek, Hazelwood MO
Staphlatex	American Micro Scan
Accu- Staph	Carr-Scarborough, Stone Mountain GA
Staph Rapid	Roche.Nutley NJ
SeroSTAT Staph	Scott Laboratories, Inc.
Veri-Staph	Zeus technologies, Raritan, NJ

II - Bound Coagulase (Clumping factor)

Duthie (1954) named the factor that converts fibrinogen to fibrin directly and does not require a coagulase reacting factor as Bound coagulase (Cheesbrough, 1985). It is responsible for the clumping associated with the slide coagulase test. It resists boiling but can be destroyed by autoclaving or proteolytic digestion. It can be detected using slide coagulase test, and it is the most widely used assay for *S. aureus* identification (Guardati *et al.*, 1993).

Many additional modified slide/tube tests have been developed and employed to distinguish *S. aureus* from other species of *Staphylococcus* (Jungkind *et al.*, 1984) these tests include:

a) Latex agglutination.

This method uses latex beads coated with plasma to detect both clumping factor, and the immunoglobulin molecules present on the beads to detect protein A (Koneman *et al.*, 1997). Several commercial Kits are listed in Table (2). Many evaluations comparing these tests have been published (Myrick and Ellnerz, 1982, Doern, 1982, Aldridge *et al.*, 1984, Pennell *et al.*, 1984, and Luijendijk *et al.*, 1996). The recommendation was in favor of Staphaurex plus and Pastorex staphplus to have excellent sensitivity for identifying *S. aureus* in clinical samples, because they showed an optimal sensitivity for identifying both Methicillin-susceptible (MSSA) and Methicillin-resistant (MRSA) strains of *S. aureus*.

b) Passive hemagglutination

The passive hemagglutination test procedures use sheep red blood cells that are sensitized with fibrinogen to detect clumping factor on the surface of *S. aureus* cells (Luijendijk *et al.*, 1996). Several products that use this approach are commercially available and include: Staphloslide (BDMS), Hemastaph (Remel) and Staphyslide (bioMerieux SA, France). Some workers prefer the passive hemagglutination procedure because a nonsensitized red blood cell suspension is included as a negative control for each test (Koneman *et al.*, 1997).

c) Immunoenzymatic assay (IEA)

It can identify *S. aureus* strains that are negative to clumping factor and protein A, which represent up to 10 - 15% of *S. aureus* isolates. The assay is based on a monoclonal antibody (Mab). Mab C1-10/11, prepared against the *S.aureus* endo-β-acetylglucosaminidase (SaG) an enzyme produced by all isolates of this species. These tests provide results more rapidly than the tube coagulase test and more accurate than the slide coagulase assay (Guardati *et al.*, 1993).

d) Other coagulase tests include:

1- StaphASE test, (bioMerieux- Vitek, Inc.), seems to be the most advanced form of the paper strip method designed by Anandam (1971). It is performed in a microcupule similar

to that used for API (Analytical Profile Index) kit. The microcupule contains dehydrated rabbit plasma. Several purified colonies are emulsified in a special medium and inoculated in the cupule. Clumping of the suspension within one minute is a positive test.

2- Fluorogenic coagulase test; is performed by inoculating a small cupule containing the substrate, which detects coagulase that reacts with prothrombin to form a complex called staphylothrombin (Manufacturer terms). The staphylothrombin then enzymatical cleavage of Catalyzed fluorogenic peptide that is present in the test cupule, causing the release of a fluorescent light. It has been demonstrated to be a highly sensitive and specific test (Janda *et al.*, 1994).

1.4.4 Deoxyribonuclease (DNase)

Cunningham *et al.*, (1956) demonstrated that *S. aureus* produces a thermostable, calcium-activated, exocellular enzyme called deoxyribonuclease. Other microorganisms, including *S.epidermidis*, elaborate similar nucleases. However, they are heat labile.

S. aureus deoxyribonuclease is a phosphodiesterase, which can cleave either DNA or RNA (Prescott *et al.*, 1990). It consists of a single polypeptide chain and a molecular weight of approximately 17000D, and the optimum pH for DNase production is 8.3 (Erickson and Deibel, 1973). The optimum temperature for the maximum enzyme production is 37°C.

Many investigators thought of using the production of DNase along with coagulase as an index of pathogenicity (Chesbro and Auborn, 1967, and Niskanen and Nurmi, 1976). Jarvis and Wynne (1969), Hoover *et al* (1983), Klapes and Vesley, (1986) suggested that the DNase test must be included in the clinical laboratory for the classification of pathogenic staphylococci, along with the coagulase test. Brandish and Willis (1970) have found a remarkable correlation between thermostable nucleases (TNase) production and coagulase formation.

On the other hand, and due to the difficulty in detecting the enterotoxins in foods, Chesbro and Auborn (1967) suggested using TNase as an indicator for the presence of enterotoxigenic strains of S. *aureus* in foods. Studies by Lachica *et al.* (1971), Lachica (1976), Niskanen and Lindroth, (1976a) and Emswiler-Rose *et al.* (1980) indicated that more than 95% of enterotoxigenic staphylococci produce heat-stable nucleases. However, Koupal and Deibel, (1978) described a rapid Qualitative method for the detection of heat-stable nucleases in foods instead of enterotoxins detection because it's easier to demonstrate.

1.4.5 Hemolysins

At least four hemolysins or cytolytic toxins are produced by staphylococci, indicated as alpha (α), beta (β), gamma (γ), and delta (δ) hemolysins. They are excreted by *S. aureus* strains together or singly (Prescott *et al.*, 1990). Almost all strains of *S.aureus* produce the alpha hemolysin, but beta hemolysin is more

commonly produced by strains from animal origin (Koneman *et al.*, 1997). They have molecular weights ranging from 21000 to 68000 Dalton.

- Alpha toxin (α)

In addition to its lethal effect on a wide variety of cell types, including its lytic effect on erythrocytes, the toxin exhibits a wide variety of other biological activities (Devriese and Hajek, 1980). It is excreted from staphylococcal cells as an inactive proteolytic enzyme that must be activated by another protease (Koneman *et al.*, 1997). It is also a potent neurotoxin and responsible for the zone of hemolysis observed around colonies of some *S. aureus* strains growing on sheep blood agar (Cheesbrough, 1985).

- Beta toxin (β)

In 1935, Glenny and Stevens described it as a separate hemolysin. A wide zone of incomplete or partial hemolysis is produced around colonies after incubation at 35°C, and becomes clear and complete at lower temperatures (Cheesbrough, 1985). This explains the so-called "hot-cold" hemolysin because its hemolytic properties is enhanced if the sheep blood agar medium was incubated at 35-37°C and followed by maintaining at 4°C (Devriese and Hajek, 1980).

- Gamma toxin (γ)

In 1930, Smith and Price described the gamma toxin. This toxin acts primarily as a surfactant, and may play a major role that causes diarrhea seen in staphylococcal food poisoning by activating the adenylate cyclase (Koneman *et al.,* 1997).

- Delta toxin (δ)

In 1947, Williams and Harper described the Delta toxin. It's a weakly specific substance active against a wide variety of cellular structures. It was found to be heat labile, and present in some strains of *S. aureus.* It's able to activate adenylate cyclase, resulting in cyclic-AMP production similar to the cholera toxin action (Koneman *et al.*, 1997).

1.4.6 <u>Exotoxins</u>

Some strains of *S. aureus* produce exotoxins including; Leucocidin which cause degranulation of the cytoplasm and destroys phagocytic leucocytes, Exfoliative toxin which is responsible for Staphylococcal Scalded Skin Syndrome (SSSS), Toxic Shock Syndrome (TSS), Enterotoxin F (TSST-1), which also cause systemic toxic shock but not emesis (Wieneke *et al.*, 1993), and other enterotoxins (Prescott *et al.*, 1990), which will be mentioned later.

1.4.7 <u>Oxidase</u>

It is an enzyme that brings about oxidation, i.e., combining

oxygen or loss of electrons in the respiratory chain (Cheesbrough, 1985). The oxidase test is used to assist in the identification of *S.aureus*, which do not produce oxidase enzymes (Koneman *et al.*, 1997), and the test is also available commercially.

1.4.8 Gram staining

A differential stain by which bacteria are classified as: Gram-positive (dark blue or violet colored) or Gram-negative (red colored) depending upon whether they retain or loose the primary stain (crystal violet), when subjected to treatment with a decolorizing agent (Collee *et al.*, 1996).

There are numerous modifications of the Gram stain; some of them are useful for staining smears of pure cultures such as Hucker modification (Pelczar, *et al.*, 1957). Others, which are valuable for preparations of body discharges such as Kopeloff and Beerman modification. However, the Gram stain does not always gives a clear-cut reaction because some organisms are Gram-variable (Harley and Prescott, 1990), and that the results must be interpreted with care.

1.4.9 Motility

There are two types of bacterial motion: Brownian movement, and true motility (Harley and Prescott, 1990). The first type results from the random motion of the water molecules bombarding the bacteria causing false movement. The true motility or self-propulsion has been recognized in many bacteria, and

involves two different mechanisms: flagellar motion, and gliding motion. The hanging drop test can examine the above types of motility or nonmotility (Benson, 1994). Nevertheless, *S. aureus* is non-motile bacteria

1.4.10 Susceptibility to antibiotics

In 1896 a 21-year-old French medical student named Ernest Duchesne discovered the penicillin, but his work was forgotten (Harley and Prescott, 1990). Thereafter, Alexander Fleming rediscovered the penicillin in 1928. He was the first to describe the antibacterial properties of penicillin, produced from a mould that he had originally misidentified (Heritage *et al.*, 1999). The discovery of penicillin stimulated the search for other antibiotics.

Many antibiotics have been discovered since then (Cephalothin, Clindamycin, Oxacillin, Cotrimoxazole, Erythromycin, Gentamicin, Oxytetracycline, Chloramphenicol, Streptomycin, Methicillin etc.). Some of them are narrow-spectrum drugs; that is, effective only against a limited variety of microorganisms. On the other hand, some are broad spectrum and attack many kinds of pathogens.

Antibiotics can be either synthesized by microorganisms or chemically semi- synthesized, which are natural antibiotics that have been chemically modified by the addition of extra chemical groups (Lim, 1998). The term of Minimal Inhibitory Concentration (MIC) is the lowest concentration of a drug that prevents growth of a particular microorganism, whereas Minimal Lethal Concentration

(MLC) describes the lowest drug concentration that kills the microorganisms.

Two methods have been used to perform the sensitivity test include: the dilution sensitivity test, and the disk diffusion test (Cheesbrough, 1985). The latter is recommended if a rapidly growing aerobic or facultative pathogen like *Staphylococcus* or *Pseudomonas* is being tested, i.e., Kirby-Bauer method recommended by the US Federal and Drug Administration (FDA) and the Subcommittee on Antimicrobial Susceptibility Testing under the National Committee for Clinical Laboratory Standards (Benson, 1994)

Staphylococci are variably sensitive to many antimicrobial drugs. Resistance falls into several classes:

1) ß-lactamase production, which is under plasmid control, that acquire the staphylococci resistance to many Penicillins (penicillin G, ampicillin etc.

2) Resistance to Nafcillin, Methicillin and Oxacillin are independent of ß-lactamase production, and the genes reside on the chromosome. The resistance mechanism is related to the lack of or inaccessibility of certain penicillin-binding proteins in the organisms (Harley and Prescott, 1990). However, The enormous sales of antimicrobial drugs and the misuse of these agents increased the number of drug-resistance pathogens (Stryer, 1988). Some of these strains could be resistant up to twenty different types of Antimicrobial agents (Masaudi *et al.*, 1988).

1.5 Staphylococcal enterotoxins

1.5.1 Definition

The staphylococcal enterotoxins are a series of proteins, produced by enterotoxigenic strains of *S. aureus* (Lim, 1998). They are found in supernatant of the culture media and in contaminated food after suitable incubation, (Sugiyama *et al.*, 1960) and cause the typical enterotoxic reaction in monkeys.

They are heat-stable and resistant to the action of gut enzymes (Koneman *et al.*, 1997), and the cells and receptors in the gut responsible for the binding of Staphylococcal Enterotoxins (SEs) have not been obviously identified (Krakauer, 1999). Also the precise mechanism of emetic effect of enterotoxin is still ambiguous (Wood *et al.*, 1997), but it is probably the result of central nervous system stimulation (vomiting center) after the toxin acts on neurological receptors in the gut (Jawetz *et al.*, 1989). The term "superantigen" is used to describe their activation of a large proportion of T cells (5-30 %) whereas, a conventional antigen stimulates less than 0.01 % of the T cell population (Krakauer, 1999).

1.5.2 Historical introduction

The staphylococci were established as the causative agent of foodborne intoxication when Dack *et al* (1930) isolated a strain of *S. aureus* from food responsible for an outbreak of food poisoning. In 1930, Jordan prepared sterile filtrates from broth cultures of this organism, fed them to human volunteers, and

observed the effect of these filtrates on humans. When an assay system was developed, he discovered that oral administration of toxic staphylococcal preparations induced vomiting in monkeys. Later, Dolman *et al.*(1936) devised the "kitten" test.

For twenty-five years, these were the only method available to study enterotoxins, until some investigators from the Food Research Institute, University of Chicago, and Food and Drug Administration, Washington, DC provided the key for understanding the nature of the enterotoxin by utilizing the tools of modern biochemistry and immunology (Casman and Bennett, 1965).

1.5.3 Composition of enterotoxins

The enterotoxins molecules are comprised entirely of only a single polypeptide chain (Bergdoll *et al.*, 1971). Fig.1 shows the Polypeptide chain to contain relatively large amounts of Lysine, Aspartic, and Glutamic acids. There are some differences in the composition of the enterotoxins including the number of amino acid and their sequence, the N-terminal amino acid and the C-terminal amino acid. Based upon amino acid identity, the enterotoxins may fall into two groups Staphylococcal Enterotoxin A (SEA), SED, SEE, SEH, and SEI in the first group (31 to 83% amino acid identity) and SEB, SEC, and SEG in the second group (62 to 64 % amino acid identity) (Munson *et al.*, 1998).

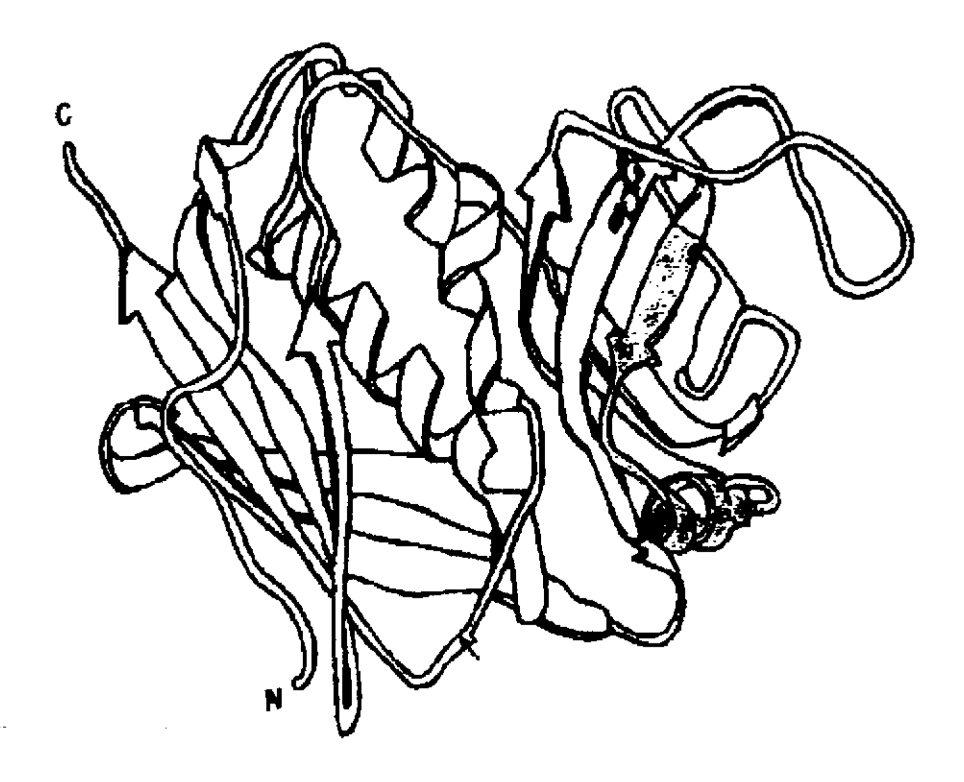


Fig. (1): A ribbon diagram of the three-dimensional of Staphylococcal Enterotoxin B (SEB) adapted from Wood *et al.*, 1997.

1.5.4 Properties of enterotoxins

The purified preparations of the enterotoxins are fluffy, snow-white colored, which are hygroscopic and readily soluble in water and salt solutions (Bergdoll *et al.*, 1959). They are resistant to proteolytic enzymes such as: rennin, trypsin and chymotrypsin. However, Pepsin destroys their activity at a pH of about 2 (Bergdoll *et al.*, 1971).

They are generally heat resistant (Niskanen and Nurmi, 1976), the crude solutions are resistant to boiling for 30 minutes, but the purified enterotoxins appear to be more sensitive to heat than the crude one (Hernandez *et al.*, 1993). Enterotoxin B appeared to be the most heat resistant of the enterotoxins (Bergdoll *et al.*, 1971).

1.5.5 Types of enterotoxins

In 1959, Casman conclusively established the occurrence of more than one immunological type of staphylococcal enterotoxin. The bases for differentiation of these proteins are their precipitin reactions with specific antibodies (Bergdoll *et al.*, 1965). Therefore, two serological different enterotoxins have been identified and suggested that the strain 196E which is associated with "food poisoning" be referred to as "type F" and the second one as "type E" because of its production by most of "enteritis" origin strains (Casman, 1959).

To provide greater flexibility and to facilitate the designation of any other staphylococcal enterotoxin that might be identified in

the future, an open meeting was held at the 62^{nd} Annual Meeting of the American Society for Microbiology to establish a system of nomenclature. It was proposed that the staphylococcal enterotoxins that had been tentatively designated types "F" and "E" be henceforth known as A and B respectively (Casman *et al.*, 1963).

Ten staphylococcal enterotoxins have been identified: A (SEA) (Casman, 1960), B (SEB) (Bergdoll *et al.*, 1959), C_1 (SEC_1) (Bergdoll *et al.*, 1965), C_2 (SEC_2) (Avena and Bergdoll, 1967), C_3 (SEC_3) (Reiser *et al.*, 1984), D (SED) (Casman *et al*1967), E (SEE) (Bergdoll *et al.*, 1971), H (SEH) (Su and Wong, 1995), G (SEG) (Munson *et al.*, 1998), and I (SEI) (Munson *et al.*, 1998). However, there is a possibility to identify more SEs (Su and Wong, 1998).

- Staphylococcal Enterotoxin A (SEA)

Casman first identified SEA in 1960. Its molecular weight is 27800 D, and has isoelectric point at pH 7.3.Under almost optimal conditions of pH, aeration, and temperature, SEA production occurs under nearly all water activity (a_w) conditions that allow growth of microorganism (Ewald and Notermans, 1988). The Nitrogen content is 16.2%, and the total number of amino acid residues is 240. The emetic dose (ED_{50}) is 5 μg/monkey. It's an extremely potent gastrointestinal toxin; as little as 100 ng is sufficient to cause symptoms of intoxication (Rasooly *et al.*, 1997). It's a leading cause of food poisoning. Markus and Silverman (1970) reported that enterotoxin A production starts heavily in the exponential phase, so this might

be the reason for it's greater frequency in causing outbreak. In general, enterotoxin secretion occurred under all conditions that permit growth of the organism (Carpenter and Silverman, 1976). Inactivation by heat is dependent on the concentration of the heated SEA (Denny *et al.*, 1971). However the serological components of purified SEA could be destroyed by autoclaving at 121.1 °C for 5 to 15 minutes (Akhtar *et al.*, 1996).

- Staphylococcal Enterotoxin B (SEB)

Bergdoll *et al* (1959) identified the second type of staphylococcal enterotoxin. It has a molecular weight of 28,366D and the isoelectric point at pH 8.6. The Nitrogen content is 16.1%, and the total number of amino acid residues is 239. The ED_{50} is 5 μg/2-3Kg monkey. It produces at the beginning of the stationary phase in larger quantities. This makes the purification of this enterotoxin easier than the others. However, after several transfers of the enterotoxin B producing culture, the production of the SEB declines rapidly in contrast to strains produce SEA. The possible reason for low incidence of enterotoxin B food poisoning outbreak is the delay in enterotoxin production during the growth phase (Markus and Silverman, 1970). Production of SEB is very sensitive to reduction in (a_w) and is hardly produced at (a_w) less than 0.93 despite the extensive growth of the organism (Troller, 1971). The production of SEB was better when pH was increased and

sodium chloride concentration was decreased (Genigeorgis and Sadler, 1966).

- Staphylococcal Enterotoxin C (SEC)

Bergdoll *et al.*, (1965) have succeeded in the identification of the third enterotoxin namely enterotoxin C. Three forms of enterotoxin C have been purified (Munson *et al.*, 1998). They have been classified as C_1 (Bergdoll *et al.*, 1965), C_2 (Avena and Bergdoll, 1967), and C_3 (Reiser *et al.*, 1984) on the basis of their different isoelectric points ranging from 8.6 to 7.0 (Noterman *et al.*, 1988). There are minor differences in the other properties, e.g., the total number of amino acid residues (296 and 294 for $C_1$and C_2, respectively), and their molecular weights ranging from 34100 to 34000D. The 50% emetic doses (ED_{50}) ranged from 5-10 μg/monkey. The results obtained by Otero *et al* (1990) indicated that both enterotoxins C_1 and C_2 were produced during either the exponential growth phase in some strains or at the beginning of the stationary phase. The effect of water activity (a_w) on SEC production follows the same pattern as with SEB (Ewald and Notermans, 1988). As the concentration of NaCl increased up to 10%, yields of SEC decreased to undetectable levels (Genigeorgis *et al.*, 1971).

- Staphylococcal Enterotoxin D (SED)

Casman *et al.* (1967) identified serologically the fourth staphylococcal enterotoxin. The production of SED alone or

with other enterotoxins by incriminated strains of food poisoning indicates that its role in food poisoning is second to enterotoxin A. Because it can be denatured very easily, and difficult to separate from other antigens; therefore, this enterotoxin is very difficult to purify (Bergdoll, 1972). The minimum amount of SED that could be detected was 1ng /ml and was produced at (a_w) of 0.86 (Ewald and Notermans, 1988).

- Staphylococcal Enterotoxin E (SEE)

Bergdoll *et al.* (1971) identified the fifth enterotoxin as enterotoxin E. The molecular weight is 29600D, and pH 7.0 for its isoelectric point. The total number of amino acid residues is 259. The ED_{50} is between 10 and 20µg/monkey.

- Staphylococcal Enterotoxin H (SEH)

Su and Lee Wong (1988) have designated a newly characterized enterotoxin H that was purified from *S. aureus* FRI-569. The molecular weight is 28500D, and the isoelectric point was estimated to be 5.7. The ED_{50} has not been yet determined but it is believed to be lower than 30 µg / monkey (Su and Wong, 1995).

- Staphylococcal Enterotoxin G (SEG) and I (SEI)

Munson *et al.*, (1998) reported two new staphylococcal enterotoxin genes were identified, and designated *seg* and *sei*. They consist of 777 and 729 nucleotides, respectively; encoding

precursor proteins of 258 (SEG) and 242 (SEI) amino acids. The molecular weight for enterotoxins G and I are 27.043 D and 24.928 D, respectively (Abe *et al.,* 2000).

1.5.6 Detection methods

1.5.6.1 Introduction

Characterization and identification of staphylococcal enterotoxins from culture media and food samples implicated in staphylococcal food poisoning outbreak have been hindered due to the low levels of enterotoxins that cause intoxication. In addition, the lack of the practical, sensitive and rapid available test methods caused more difficulties in detecting even the unidentified enterotoxins.

1.5.6.2 Biological methods

These were the first methods used by the early investigators, which methods provide suitable means to detect the existence of undiscovered enterotoxins (Bergdoll *et al*1965), On the other hand, these methods cannot differentiate between the different types of enterotoxins (Su and Wong, 1998). It also requires special facilities, which are very difficult to provide in all concerned laboratories. Of the various animals that have been tested for sensitivity to enterotoxins, monkeys (Jordan and Broom, 1931) and cats (Dolman *et al.*, 1936); chimpanzees were the most sensitive (Wilson, 1959). The monkey-feeding test is more recommended than Cats test because the latter gives variable to unreliable results. Cats are also relatively insensitive to enterotoxin

C (Bergdoll, 1972). The monkey- feeding test utilizes six young rhesus monkeys that weigh 2-3 Kg for each sample. Samples are administered in solution, 50 ml via a stomach tube, then the animals should be observed for at least 6 hours. Vomiting by at least two animals was accepted as a positive reaction of the enterotoxin.

1.5.6.3 Immunological Techniques

Most specific and sensitive tests available for enterotoxins are based on the reaction of the enterotoxins with its specific antibody. Highly potent specific antisera can be prepared in rabbits, then used in vitro. Many tests have been developed, and therefore, it could be easier to handle them in the following groups:

1. The first group contains the gel diffusion precipitin reaction tests. The first test in this section is the single gel diffusion test; in this test a solution containing an antigen is placed over a semi-solid agar column containing its homologous antiserum. The second method called the double gel diffusion test. This was developed by Oakley and Fulthorpe (1953). In 1958 Ouchterlony devised a double gel diffusion test that utilizes an agar plate. Followed by slight modification by Wadsworth in 1957 who utilized a thin layer of agar on an ordinary microscope slide and named it microslide double gel diffusion test (Casman *et al.*, 1969). Afterwords, Gandhi and Richardson (1971) developed other diffusion assay

methods based on more economy in using reagents, greater specificity, and simplicity in performance. They named it the capillary tube immunological assay. In 1980, Meyer and Palmieri developed and tested a polyvalent Single Radial immunodiffusion (SRD) method, which can be used with a multiple-culturing procedure for screening large numbers of food or other isolates. The technique of electro-immuno-diffusion introduced by Laurell (1966). This was combines the benefits of both electrophoresis and immunodiffusion techniques. The second group includes, Passive Hemagglutination Assay (PHA) (Johnson *et al.*, 1967), and Reversed Passive Hemagglutination Assay (RPHA) (Silverman *et al.*, 1968). The latter method can detect enterotoxins in clinical samples or in food without concentration of the extracted enterotoxin; since the extraction of the enterotoxins from foods is laborious and time consuming (Genigeorgis and Kuo, 1976). This method shows occasionally nonspecific agglutination reactions with certain types of food, so Saloman and Tew (1968) replaced red blood cells with polystyrene latex particles, and called it Latex agglutination assay. Furthermore, Shingaki *et al.* (1981) improved the test using highly purified anti-enterotoxins prepared by affinity chromatography, and called it Reversed Passive Latex Agglutination test (RPLA). The commercial kits become

widely used, and show high specificity and sensitivity with a detection limit as minimum as 0.75 ng enterotoxin per gram of food. The disability of the kits for detection of SEE, SEH, SEG and SEI does not create a great problem as they seldom occur (Wieneke, 1988). Also, about 5% of food-borne staphylococcal outbreak are caused by SEE, SEH, SEG and SEI and unidentified SEs (Su and Wong, 1995). Therefore, the RPLA test still the best.

2. The third group includes methods that use some material (such as dyes, radioactive elements, and enzymes) to label antibody molecules. However, Coons and Kaplan (1950) were the first to show that antibody molecules could be labeled by conjugation with fluorescent dye, and then used the labeled antibody to detect a specific antigen in cells. The labeled antibody under ultraviolet light emitted a yellow-green fluorescent that could be viewed in a specially constructed microscope. Radio-immuno -assay test (RIA), also detects a specific antigen using radioactive iodine (I^{125}) rather than fluorescein (Niskanen and Lindroth, 1976b). This method detects enterotoxins in clinical samples or in food (Orth, 1977), and can be made within one working day once the labeled enterotoxins are available (Miller *et al.*, 1978). Enzyme-Linked Immuno-Sorbent Assay (ELISA) Freed *et al.*, 1982 and Meyer *et al.*, (1984) used the enzyme immunoassay, which was

developed for detection of SEs in foods. The ELISA can be completed as quickly as the RIA. It is sensitive but does not require the use of radioactive material. Two types of ELISA methods have been proposed. In the "double-antibody sandwich" method, the enzyme is coupled to the specific antibody, whereas in the second method, the enzyme is coupled to the enterotoxin (Christensson *et al.*, 1984).

1.5.6.4 Molecular Methods

Several modern molecular and genetically developed techniques have been used to identify staphylococci, and to characterize strains in epidemiological studies, and in outbreaks of unusual or multiresistant strains. These methods include:

1- Whole-cell protein using SDS-Polyacrylamide Gel Electrophoresis (PAGE) (Sugai *et al.*, 1990).
2- Restriction enzyme fragment length polymorphism analysis of plasmid, ribosomal, and chromosomal nucleic acids (Prescott *et al.*, 1990).
3- Pulsed-inversion Gel Electrophoresis of total DNA (PIGE) (Tenover *et al.*, 1994), and Pulsed-field Gel Electrophoresis (PFGE) (Khambaty *et al.*, 1994)
4- Polymerase Chain Reaction (PCR), and more newcomers, and quick display will be about the latest technique.

- Polymerase Chain Reaction (PCR).

The PCR is the most far-reaching development in molecular techniques during the last decade (Smith, 1996). The PCR was first patented in 1987 and then was commercialized in 1988 by American Cetus Corporation. The PCR procedures involve denaturation, annealing and extension using DNA polymerase. The completion of these three processing steps comprises a cycle and can be done in automated thermal cyclers (PCR machines). The three processing steps start by heating the double-stranded DNA at 95-98°C in order to separate them into two single strands. The synthetic oligonucleotide primers then bind to their complementary sequence and are extended in by DNA polymerase in the presence of all four deoxynucleoside triphosphates, giving a new strand of DNA that is identical to the template's original partner (Wilson *et al.*, 1991).

These methods have gained widespread popularity as highly discriminatory and relatively inexpensive methods for epidemiologic typing of strains.

1.6 Sanitation in food handling

1.6.1 Food handlers

The WHO used the "food-handling personnel" term to identify those who may come into contact with part or all of an edible end product at any stage from its source (WHO, 1989). This includes an inspector who, in his routine work, comes into direct

contact with the food itself. This is in general, but a distinction has been made between those whose work could allow transferring pathogenic organisms from themselves to food in such a way that illness might result (Cruickshank, 1990), in addition to those for whom such risks does not exist. Those who possibly present a risk can be defined as food handlers and include those whose work involves touching unwrapped foods to be consumed raw or without further cooking or other forms of treatment, i.e., people involved in such activities as the preparation of salads, sandwiches, and cook food (WHO, 1989).

1.6.2 Food handlers as source of *S. aureus*

Food handlers play a significant role in ensuring food safety throughout the chain of food production. Mishandling and disregard of hygienic measures on their part may enable pathogens to come into contact with food (Bidawid *et al.*, 2000), and such pathogens may survive and multiply in the food and subsequently cause disease.

The human nose, hand and skin are the main habitats of *S.aureus*. They reside in the mucous membranes of the nose, and they can penetrate into the deeper layers of the skin, where they live in the pores and hair follicles and multiply. Nasal secretions contain large numbers of bacteria including large proportion of staphylococci. Around 30-50% of humans carry staphylococci in their nose. Whereas, in patients the nasal carriage may be as high as 60- 80% (Hobbs and Roberts 1990).

Food handler's hands carry the major responsibility in transferring pathogens to food. The habits of fingering (picking)the nose and spitting on the ground will also increase the hazard of passing the staphylococci from the food handler's hands to food. The pus from skin lesions, i.e., carbuncles, septic cuts and burns contains innumerable organisms, and a small speck of pus could inoculate food with millions of staphylococci. The manner of spreading of staphylococci from human reservoir to food is suggested in Fig.2.

In many incidents of staphylococcal intoxication, the same phage typing and enterotoxin-producing types of *S. aureus* were isolated from both food handlers and implicated food (Hobbs and Roberts, 1990), (Bidawid *et al.*, 2000).

More often, the food handlers become either infected due to frequent contact with contaminated raw foods, tasting or eating left-over contaminated food, so that they are victims too.

1.6.3 Rice dishes

Nowadays, rice dishes become one of the everyday dishes of the Saudi's family. In restaurants it is regarded as the primary dish of the menu. Many reasons could be responsible for their popularity include:

- It is easy to cook and eat
- It is light in digestion
- It is delicious

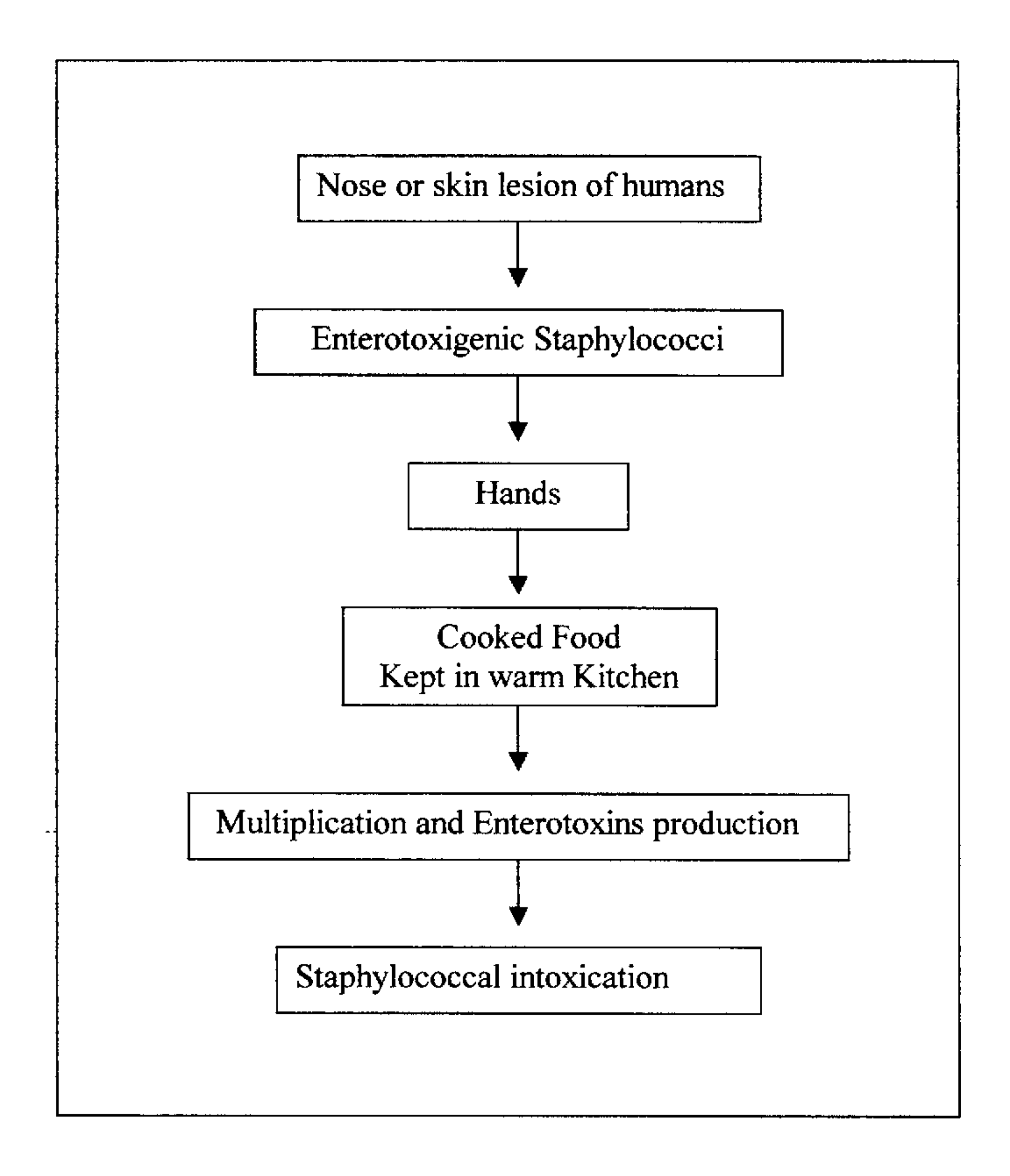


Fig. (2): Spreading of staphylococci from human reservoir to food

In the past, Saudis were depending on bread as a major staple of carbohydrate, while at present they called rice dishes as the king of the menu (main course dish). In many dishes, rice is one of the secondary ingredients, i.e., mahshy. On the other hand, it can be one of the primer ingredients of the meal with either white or red meat (fish and birds or lamb, beef and camel respectively). In Saudi Arabia there are many popular meat and rice dishes presented in Table (3)

1.6.3.1 Distribution system

Today, retailing of cooked food covers many different outlets, which range from the tiny corner-cafeteria to large restaurants. The cooked rice is usually kept at temperature not less than 62°C in hot cabinets or in the pot used in cooking process over mild source of heating. The cooked foods act as vehicles of intoxication when the pot or the hot cabinets were held at inappropriate temperature. Therefore, they may serve as incubator, and encourage the growth of microorganisms; especially when they receive much handling during preparation and distribution. Thus, the purchaser may obtain food already contaminated with microorganisms. Also, further helding and storage at wrong warm temperature will enhance bacterial multiplication to levels resulting in food poisoning.

1.6.3.2 Left-over and it's relation to incidences of intoxication

In normal time in domestic kitchens, it is easy to arrange for preparation and cooking so that a meal can be served directly hot. However, during high seasons, i.e., (Hajj) when large numbers of people need to be fed in a short period of time; It is often expected to prepare foods hours before it is needed. So that the cooked foods are held and placed in a warm- holding apparatus at temperature of at least 63°C, or stored below 10°C, and maintained at these temperatures until needed.

The number of outbreaks of food poisoning will significantly increase with foods that were left for long time at ambient temperature. Nevertheless, when the left over from previous batches is served at first and held in hot-holding equipment and not preheated by the addition of hot gravy or sauce (Hobbs and Roberts, 1990).

Table (3): The famous rice dishes in Saudi Arabia

No.	Rice Meal	Ingredients
1	Rozabyad	Boiled rice without any additives
2	Saleq	Meat with rice and milk
3	Madeny	Meat, rice, sprinkled raisin, Pine and almonds
4	Arabi	Meat, rice, and Butter (Baladi Ghee)
5	Kably	Meat marinated in paste of garlic, chopped onions, spices, and saffron with kady flavor (Pandanus tectorus)
6	Rozoadas	Lentils with rice served with fried dry fish
7	Sayadah	Rice with Cinnamon served with cooked Fish
8	Rozohomus	Meat with rice mixed with splet checpeas
9	Burriany	Same as Kably Ingredients with tomato and tomato paste and different spices
10	Mandy	Meat cooked in clay oven and rice cooked in separate pot located underneath the meat so that the released fat and juices sip into the rice pot. Sometimes, turmeric may be added
11	Haneez	Same as Mandy with only one difference, which is the adding of markh (*Alyssum homalocarpum*) as flavor enhancer
12	Bokhary	Meat marinated in paste with tomato and tomato paste, garlic, chopped onions, and different spices with chopped carrots and raisins
13	Koze	The same as Bokhary but, with added macaroni and different spices
14	Madoghott	Same as Bokhary but with the use of pressure pots scientifically, it sterilizlized

Materials and Methods

2.1 Specimens, sampling, media, and culture conditions

2.1.1 Food handlers specimens

Clinical specimens were collected since 1999 from foodhandlers of different nationalities, who applied to work in hospital-located kitchens in Makkah. The specimens, which included: nasal swabs, throat swabs, nail swabs, stool samples and wound swabs were examined for presence of *Staphylococcus aureus*. Ready-made Amie's medium was used to transport these specimens to the laboratory within five hours. These specimens were sub-cultured directly on 5% sheep blood agar and mannitol salt agar. Any morphologically suspected colonies of *S. aureus* were examined using coagulase test, and then confirmed by Catalase test, Gram staining, and DNase test. Reversed Passive Latex Agglutination test (RPLA) was used to evaluate the ability of all isolates to produce enterotoxins A, B, C, or D.

2.1.2 Preparation of food

2.1.2.1 Bokhary Rice

There are about half-a dozen most popular variations of the classic Bokhary rice dishes all over Saudi's regions, each one with its own name, ingredients, method of cooking and method of serving (Salah, 1977). It is prepared with lamb or chicken. Meat, chicken or lamb is first marinated for at least 1.5 hrs in a condiment paste of garlic, chopped onions, lemon juice, tomato and tomato

paste, and spices. Then the cleansed washed rice is mixed with the condiment. Chopped carrots, small amount of sugar, and raisins are sprinkled on the top of the pot, which was covered with the lid. Rice and meat mix continue to cook over low heat for more than thirty minutes until well cooked. The meal is then ready to serve (Salah, 1977).

2.1.2.2 Mandy Rice

Mandy is perhaps one of the most elaborate rice dishes. The method of cooking Mandy is known as mandaia. Mandy is the best way of cooking lamb, and the cooked meats are believed to be exceptionally tender. It is cooked with lamb but sometime with chicken. It is prepared using a large clay oven above the ground or in a hole in the ground, which is lined with clay bricks or sometimes; an opened petroleum barrel is used. This method is very popular. Burning wood is placed at the bottom of the hole, and then a large bowl of rice is lowered on top of the firebrand (burning charcoal). A whole lamb is then placed horizontally or vertically on a steel wire mesh (a steely spit) in the oven, (see Fig. 3) and a heavy lid is placed on top to hold the steam from escaping. However, the lid is sometimes covered with sand.

As the lamb slowly cooks in the hot oven it releases fat and extract, which fall into the pot of the rice. After two to three hours of cooking a matter of judgment on the part of the cooker

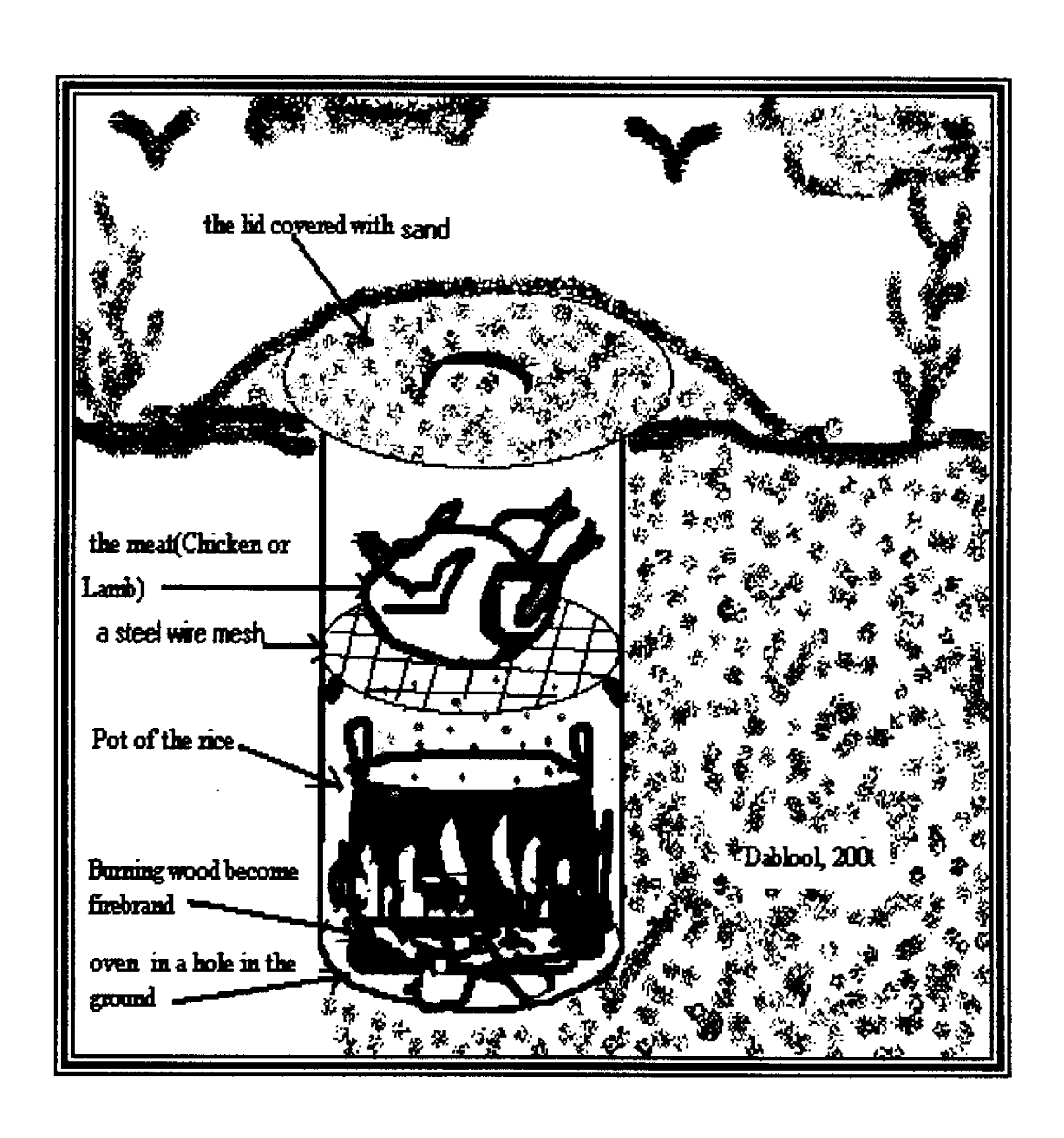


Fig. (3): Underground oven for Mandy.

experience according to meat type and age of the carcass, then the sealed lid is removed and the Mandy is ready to serve.

2.1.3 Sampling of Mandy and Bokhary rice

Rice samples in these experiments were obtained from six different restaurants in Makkah. Restaurants were designated as: R1, R2, R3, R4, R5, and R6. The weight of each sample was approximately 500g. To count the bacteria, which could contaminate the main pot during the day, the total plate counts were done; by using plate count agar and results were obtained after 24 hrs incubation at 35°C, and 55°C. Samples were taken at noon when the restaurants begin to open to the customers to serve lunch and also ten hours later at dinner time. To study the favorable handling and holding conditions encouraging toxins production, autoclaved rice samples were used, and inoculated with the enterotoxigenic strain.

2.1.4 Media

Five kinds of media were used in this study: Transport medium, isolating media, DNase test medium, enterotoxin production media, and sensitivity test medium (The Oxoid Manual, 1982).

1) Transport medium:

The specimens were obtained using sterile swabs supplemented with ready-made transport Amie's medium. It was used to preserve the viability of the bacteria

Table (4): Composition of Amie's medium.

Component	Gram per liter
Sodium chloride	3.0
Sodium hydrogen phosphate	1.5
Potassium dihydrogen phosphate	0.2
Potassium chloride	0.2
Sodium thioglycollate	1.0
Calcium chloride	0.1
Magnesium chloride	0.1
Agar No.1	4.0
pH	7.2+/-0.2

including *S. aureus* during transportation. The composition of Amie's medium is given in Table (4).

2) Isolating medium:

Plate count agar medium was used for bacterial total counts (the composition of this medium is given in Table (5), and other three types of media were used to isolates *S. aureus* from clinical specimens, and food samples. The first medium was 5% sheep blood agar, a non-selective medium (Finegold and Sweeney, 1961). The composition of sheep blood agar medium is given in Table (6). The second one was a mannitol salt agar (Niskanen and Aalto, 1978), which is a differential and selective medium used to isolate *S. aureus* from faecal specimens (Cheesbrough, 1984). The composition of mannitol salt agar medium is given in Table (7). The Baird-Parker medium was the third one used to isolate *S. aureus* from food samples (Devriese, 1981) and from clinical specimens (Lachica, 1980). The composition of Baird-Parker medium is given in Table (8). The replacement of Egg Yolk (EY) with Tween 80 and $MgCL_2$ provides several advantages such as:

- Avoiding EY variability.
- The absence of (EY) reduces the outgrowth of other contaminating bacteria.
- Reduces the cost and handling of the medium during preparation (Lachica, 1984).

Table (5): Composition of Plate Count agar medium.

Component	Gram per liter
Tryptone	5
Yeast extract	2.5
Dextrose	1
Agar	9
pH	7.0+/- 0.2

Table (6): Composition of blood agar base No.2.

Component	Gram per liter
Protease Peptone	15.0
Liver Digest	2.5
Yeast Extract	5.0
Sodium chloride	5.0
Agar	12.0
pH	7.4-/+0.2

5% Sheep blood (sterilized) added

Table (7): Composition of Mannitol Salt Agar medium.

Component	Gram per liter
Lab-Lemco powder	1.0
Peptone	10.0
Mannitol	10.0
Sodium chloride	75.0
Phenol red	0.025
Agar	15.0
pH.	7.3 - 7.7

Table (8): Composition of Baird- Parker medium.

Component	Gram per liter
Lab-Lemco powder	5.0
Tryptone	10.0
Yeast Extract	1.0
Sodium pyruvate	10.0
Glycine	12.0
Lithium chloride	5.0
Agar	20.0
Tween 80 (modification)	0.05%
$MgCl_2$ $6H_2O$ (modification)	0.1%
pH	6.8-/+0.2

Table (9): Composition of DNase medium.

Component	Gram per liter
Tryptose	20
Deoxyribonucleic acid	2
Sodium chloride	5
Agar	12
pH	7.3+/- 0.2

Table (10): Composition of Mueller Hinton Agar medium.

Compound	Gram per liter
Meat infusion	6.0
Casein hydrolysate	17.5
Starch	1.5
Agar No.1	10.0
pH	7.4 -/+ 0.2

3) DNase test medium.

Deoxyribonuclease medium was used to perform DNase test, and the composition is given in Table (9).

4) Sensitivity test medium.

Mueller Hinton Agar is now widely used for sensitivity test. Clear zones of inhibition are evident and could be measured. The composition of Mueller Hinton Agar is given in Table (10).

5) Enterotoxin production media:

Two types of media were used to evaluate the enterotoxigenicity of the isolates. The first one is Brain Heart Infusion (BHI) (Casman and Bennett, 1963). The composition is given in Table (11). The second medium is Tryptone Soy Broth medium (Doorne *et al.*, 1982), and the composition is given in Table (12)

2.1.5 <u>Reagents</u>

Many reagents have been used to identify *S. aureus* in this study including:

- Phosphate-Buffered Saline Solution (PBS).

Ten tablets of Phosphate buffered salts (modified Dulbecco's formula, cat. No. 28-103-05,made by Flow Laboratories, U.K) were dissolved in 1000ml distilled water and autoclaved for 10 min at 115°C. The solution was quite free from insoluble matter. The composition of this saline is given in Table (13).

Table (11): Composition of Brain Heart Infusion broth (BHI).

Component	Gram per liter
Calf brain infusion solids	12.5
Beef heart infusion solids	5.0
Proteose Peptone	10.0
Sodium chloride	5.0
Dextrose	2.0
Disodium phosphate	2.5
pH	7.4 -/+ 0.2

Table (12): Composition of Tryptone Soy Broth (TSB).

Component	Gram per liter
Pancreatic digest of casein	17.0
Pipaic digest of soybean meal	3.0
Sodium chloride	5.0
Dibasic potassium phosphate	2.
Dextrose	2.5
pH	7.3-/+0.2

Table (13): Composition of Phosphate-buffered saline (PBS).

Compound	Gram per liter
Sodium chloride	8.0
Potassium chloride	0.2
Disodium dihydrogen phosphate	1.15
Potassium dihydrogen phosphate	0.2
pH	7.3 -/+ 0.2

Table (14): Composition of 1% Peptone Water.

Compound	Gram per liter
Peptone	10
Sodium chloride	5
pH	7.2 -/+ 0.2

Table (15): Composition of REMEL's rabbit plasma.

Compound	Per one liter
Sodium chloride (CAS7647-14-5)	4.5g
Rabbit plasma w/EDTA	500ml
Demineralized water (CAS 7732-18-5)	500ml

- Peptone Water (diluent solution).

To make serial dilutions for total bacterial counts, the samples were homogenized in 1% peptone water (Rodriguez *et al.*, 1996) see Table (14).

- Egg Yolk-Tellurite Emulsion (Oxoid SR54).

Fifty ml of the emulsion was added to 1 liter of Baird-Parker medium CM275. The 50 ml of the emulsion contain the equivalent of 3ml of 3.5% potassium Tellurite, (i.e., concentration in SR54 is 0.21% w/v). It is advisable to wait for few seconds to allow the insoluble particles to settle before addition to the medium. The prepared Petri dishes can be stored in refrigerator, to be used for a maximum period of 7-10 days (The Oxoid Manual, 1982).

- Coagulase Plasma.

The REMEL's rabbit plasma is dissolved in Ethylene Diamine Tetra Acetic acid (EDTA) as recommended by the manufacturer. The formula of this reagent is shown in Table (15). The kit contains 6 bottles of the lyophilized coagulase plasma.

- Staphaurex.

The Staphaurex reagent consists of polystyrene latex particles, which have been coated with fibrinogen and IgG. The kit contains three bottles of the test latex suspension, disposable reaction cards, and disposable sampling and mixing

sticks. Each bottle of the latex test contains a minimum amount of 1.7 ml of the reagent.

- Hydrogen Peroxide 3% (V/V)(H_2O_2).

Hydrogen Peroxide solution (N. Avondale Laboratories Limited. England) was prepared. The reagent was Shaked before use. This will help to expel any dissolved oxygen, which may give false results.

- DNase reagent (Hydrochloric acid, 1 N).

The *S. aureus* colonies were tested for DNase production by flooding the plate of DNase medium with 1 N hydrochloric acid solution. To make 100 ml stock solution, fill half of a 100ml volumetric flask with distilled water, add 8.6ml of concentrated hydrochloric acid, make up to the 100ml volume mark with distilled water, transfered to labeled bottle, and stored at room temperature.

- Oxidase reagent.

The Oxidase reagent was prepared fresh before use, since it is not stable. 100mg of Tetramethyl-ρ phenylenediamine was dissolved in 10 ml distilled water (Cheesbrough, 1984).

2.1.6 <u>Culture purification and maintenance</u>

The cultures were checked for purity, by subculturing several times on selective medium, i.e., Baird-Parker medium. The streaking

method was used to produce good spacing between colonies. One colony was picked, and inoculated into 1% peptone water (diluent). Three dilutions were made. Then 1ml was spread on Baird-Parker medium, incubated at 37 °C for 24h. Colonies were checked morphologically and microscopically for purity.

All identification tests were re-checked, and finally, subcultured on nutrient slants. After incubation, slants were stored in a refrigerator, and transfered every two weeks. However, for long-term preservation, all isolates were stored at -20°C sterilized glycerol (Cheesbrough, 1984).

2.2 Morphological studies

2.2.1 Morphological and pigmentation studies of colonies.

Colony morphology and pigmentation was observed on 5% sheep blood agar, Baird-Parker medium (Hoover *et al.*, 1983) and DNase test medium (Devriese and Hajek, 1980).

2.2.2 Haemolysis

All isolates were grown on blood agar plates containing 5% sheep blood and checked for any haemolysis (Hoover *et al.*, 1983). Streaked cultures were examined after 24h of incubation at 37 °C. The term α, β, γ, and δ haemolysis should not be used with *Staphylococcus* (Devriese and Hajek, 1980). Because, any sign of haemolysis is regarded as positive result.

2.2.3 Gram stain

Smears for Gram stain were prepared from 24 hr cultures (Freney *et al.*, 1999). Hucker's modification method was used and the smears were examined for cell morphology, staining properties, and aggregation patterns (Pelczar *et al.*, 1957).

2.2.4 Motility

Cultures grown on solid media were examined for motility test using the hanging drop technique, and observed with a bright field microscope with oil immersion lens (Freney *et al.*, 1999).

2.3 Biochemical Studies

2.3.1 Catalase test

According to Bergey's Manual of Bacteriology 9th edition (Holt *et al.*, 1994) the *Genus* Staphylococcus is differentiated from the *Genus* Streptococcus by the presence of catalase enzyme. The latter converts hydrogen peroxide to water and nacent oxygen (Jensen and Hyde, 1963). To determine whether catalase is produced or not, Place few drops of 3% hydrogen peroxide (H_2O_2), on a slide and with a wooden applicator stick, pick only the top of the colony carefully in order to avoid carryover of blood, from the medium which may give false-positive reaction (Freney *et al.*, 1999). Any bubbling is recorded as positive catalase reaction (Martin *et al.*, 1976).

2.3.2 Coagulase test

Slide and tube coagulase tests have been employed to distinguish *S. aureus* from other species of *Staphylococcus* (Jungkind *et al.*, 1984). For many years, the tube coagulase test which detects the production of free coagulase was considered the ``gold standard`` (Luijendijk *et al.*, 1996). However, the limitation of this method have been addressed in various reports (Baker *et al.*, 1985). Neverthless, rapid slide agglutination test, that described by Essers and Radebold (1980) has been used. It is a modification of the slide coagulase test, and has the advantage of identifying *S.aureus* immediately instead of after a period ranging from 4 to 24 hrs with tube Coagulase test (Jungkind *et al.*, 1984).

- The rapid slide agglutination test

  The test was performed according to the procedure recommended by Murex Biotech (manufacturer). The latex bottle was first shaken to obtain an even suspension. One drop of the latex reagent was placed into a circle on the reaction card for each culture to be tested, then one colony was picked up using the disposable wooden stick (a colony was touched with the flat end of the stick). However, it was mixed with the dispensed drop of the latex reagent. The card was then rotated gently by hand for up to twenty seconds . A positive result was indicated by the development of an agglutination pattern.

In case of any uncertain results occurred; the culture needs to be confirmed by the tube coagulase, because some strains defficient in clumping factor usually produce free Coagulase (Rhoden and Miller, 1995).

- The tube Coagulase test

  This test was performed according to the procedure recommended by the manufacturer. It includes resuspension of the lyophilized coagulase plasma with sterile distilled water according to the volume size indicated on the vial. 0.5ml of the prepared plasma was added to test tube. Then a large loopful of purified colony grown on agar plate was inoculated into the tube. The tube was vortexed thoroughly to suspend the cells. Later, the tube was incubated at 35-37°C for up to 24 hrs, and observed every 30 minutes. Any visible clot observed could be considered as positive result. (The test should not be performed from media having a high salt content e.g., mannitol salt agar)

2.3.3 <u>Oxidase test</u>

Oxidase activity is estimated by dropping 1% tetramethyl-ρ-phenylenediamine dihydrochloride on a piece of whatman filter paper No.2 in a Petri dish, and a loopful of culture was picked and smeared over a small area of the filter paper (Benson, 1994). A blue-violet color, which appears within few seconds, is considered as a positive result. All *Staphylococcus* species are oxidase-negative

except for strains of *S. caseolyticus*, *S. sciuri*, *S. lentus*, and *S.vitulus* (Koneman *et al.*, 1997).

2.3.4 DNase test

This test is used to differentiate *S. aureus*, which produces the enzyme Deoxyribonuclease (DNase) from other staphylococci that do not produce it (Hoover *et al.*, 1983). The composition of this medium is shown in Table (9). The test organism was cultured on a DNase medium. After overnight incubation the plate was flooded with normal hydrochloric acid. Within 5 minutes, DNase producing colonies were surrounded by clear area and formed a cloudy precipitate containing nucleotide fractions, which indicate DNA hydrolysis (Cheesbrough, 1984).

2.4 Sensitivity test

The recommended medium in this test is Mueller Hinton agar, and Kirby-Bauer test method was used (Benson, 1994).

In this study, the control strain (*S. aureus* ATCC-25923) was inoculated on a separate Petri plate medium, so that the results are comparable. The inoculation of the medium's surface with the test organism was made with a cotton swab from broth culture standardized to 0.5 MacFarland. The turbidity of standard 0.5 MacFarland is equivalent to an overnight incubation of one colony in the broth culture medium.

Multi-disks antibiotics (previously warmed to room temperature) were place on inoculated medium by sterile forceps

Table (16): The limits of inhibition zones for Gram (+) Bacteria

Antimicrobial Agent	Abreviation	Disk potency	Disk color	Zone diameter (mm)		
				R*	MS*	HS*
Cephalothin	KF	30 µg	**Primrose**	**<14**	**15-12**	**>18**
Clindamycin	CD	2 µg	White	**<14**	**15-20**	**>16**
Oxacillin	OX	5 µg	White	**<10**	**11-12**	**>13**
Cotrimoxazole	TS	25 µg	White	**<10**	**11-15**	**>16**
Erythromycin	E	15 µg	Red	**<13**	**14-22**	**>23**
Gentamicin	GM	10 µg	Salmon	**<12**	**13-14**	**>15**
Oxytetracycline	OT	30 µg	Brown	**<14**	**15-18**	**>19**
Penicillin G	PG	10**units**	Pink	**<28**	**-**	**>29**

* (R) Resistant , (MS) Mild sensitive, (HS) High sensitive.

and each disk was pressed slightly to ensure close contact with the medium. The Plate was then incubated aerobically overnight at 37°C. Therefore, the radius of the inhibition zones was measured (to the nearest millimeter) from the edge of the disk to the edge of the inhibition zone.

Size of inhibition zones of all isolates were compared with the size of the inhibition zones for *S. aureus* (ATCC-25923), which is close to the standard zones given in Table (16), which was supplied with the disks kit from Mast Group Ltd.

2.5 Bacterial load of cooked rice during holding time

Standard plate count method was used. Twenty-five grams from rice sample was diluted with 225ml of 1% pepton water (1/10 dilution). Other dilutions were made to obtain colony counts ranging between 30 and 300 colonies (Raj and Liston, 1961).

2.6 Growth curve determination

The growth curve was determined by measuring the turbidity of the bacterial population using Brain Heart Infusion broth (BHI). This method measures the biomass, which could be correlated with density (Harley and Prescott, 1990). This indirect method uses spectrophotometric measurements of the developing turbidity, by employing samples of the test culture taken at one hr interval to serve as an index of increased cellular mass. The generation time (g) was calculated using the following equation:

$$g = \frac{0.301t}{\log_{10} N_t - \log_{10} N_0}$$

Where N_0 is the number of bacteria at point (a) or any other point at the beginning of the log phase, N_t is the number of bacteria at point (b) or any other point near the end of the log phase, t is the time in minutes between (b) and (a).

2.7 Enterotoxins studies

2.7.1 Enterotoxin production in culture medium

Each isolate of *Staphylococcus aureus* was cultured individually in Brain Heart Infusion broth (BHI), and incubated at 37°C for 18-20 hrs. Thereafter, the culture was centrifuged at 3000 rpm for 20 minutes, and the supernatant was used in the test (Manufacturer method).

2.7.2 Enterotoxin production in food

A selected isolate was inoculated in Tryptic Soy broth, and incubated at 35°C for 48h. The culture was then centrifuged at 3000rpm for 20 minutes washed twice with Phosphate-buffered saline (PBS), resuspended in saline, and adjusted to about 10^6cells/ml using a spectrophotometer (LKB Ultrospec 4050 No. 40003511. Biochrom. Cambridge) at 600nm. Ten ml of bacterial cell suspension was inoculated into specimens rice (100g of autoclaved ready-made Mandy rice or Bokhary rice) and incubated

at both 25°C and 40°C (the temperature of the incubation was recorded by an automatic recording thermometer, model: No.515P, 7days, Pacific Transducer Corp. USA). Mandy or Bokhary rice was sampled every hour, until the toxin presence was detected using RPLA technique (see section 2.7.4). Baird-Park agar was used for *S. aureus* counts. This is the method of Park and Szabo (1986).

2.7.3 Food samples preparation for RPLA

Two methods were used for the food sample preparation:

1-The Denka Seiken Co Ltd., Tokyo kit procedure, which includes blending of 10g of the food sample with 90mL of Phosphate-Buffered Saline (PBS) for homogenization using Stomacher (Seward Medical UAC House, London, U.K.), followed by centrifugation at 3000 rpm for 20 minutes. The supernatant was used for enterotoxin detection.

2- Procedure of Park and Szabo (1986)

This method includes blending of one volume of the food sample in two volume of (PBS). The food was homogenized using Stomacher and was kept for 10 min at room temperature. Then, samples were centrifuged at 4000 rpm at 4°C for 60 min (IEC Centra MP4R, Refrigerant type HP-80 by International Equipment Company, USA). The supernatant was used for enterotoxin detection.

2.7.4 RPLA test in liquid culture

The kits were obtained from Denka Seiken Co Ltd., Tokyo. The contents include four vials of control lyophilized, purified staphylococcal enterotoxins A, B, C and D (each vial contains 0.5ml), four vials of a suspension of latex particles sensitized with the corresponding anti-enterotoxins, a control latex solution, and two vials of 50mL diluent solution.

The test was performed by the procedure recommended by the manufacturer as follows.

i. 25μl Diluent was placed into each of the five rows of microtiter wells of the microplate (V-type).
ii. 25μl of the sample (food or culture) was added to the first well of each of the five rows and mixed thoroughly.
iii. Serial twofold dilutions were made by transferring 25μl of the mixture from the first well to adjacent well and repeated until the last well of the row.
iv. The reagents of the latex particles sensitized with anti-enterotoxins A, B, C, D and the Control latex were shaken, and 25μl of sensitized latex anti-A was placed to all wells in the first row, 25μl of sensitized latex anti-B was placed to all wells in the second row, and the same procedures were repeated with anti-C, anti-D, and Control latex to the wells of the third, forth, and fifth row, respectively.

v. The microplate was shaken by hand in circular manner. Then covered with Para-film to avoid evaporation of the solutions in the wells or kept in a moisture box, and left at room temperature for 18-20 hours.

vi. After incubation period, the microplate wells were observed to see whether agglutination (Fig.4) occurred or not by looking from above the microplate and against a black background. Fig.5 shows all different type of negative or positive results that observed using RPLA method.

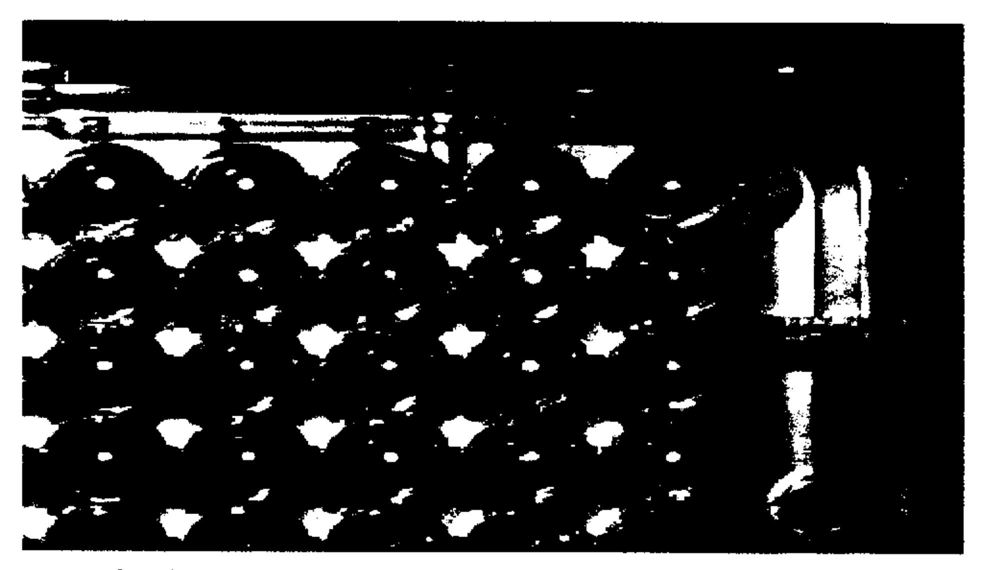


Fig. (4): Agglutination patterns of the RPLA test

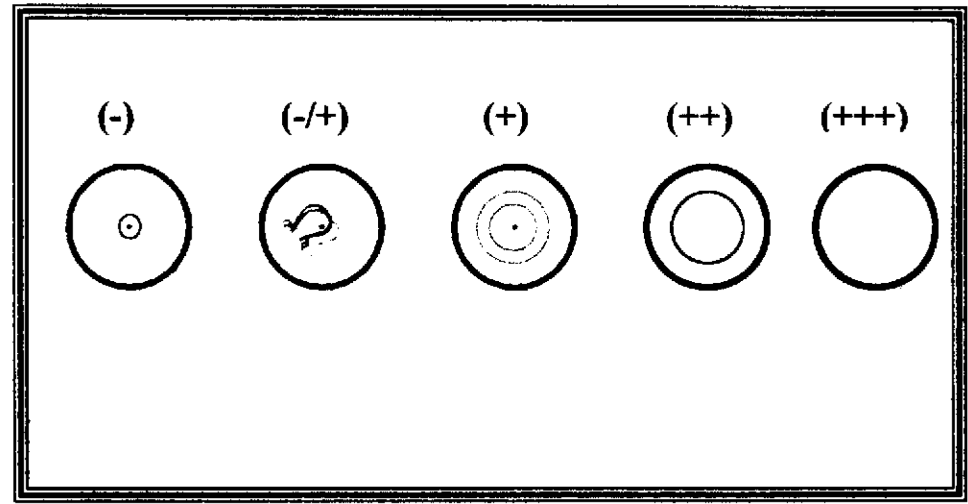


Fig. (5): Agglutination patterns of the RPLA test result

Results and Discussion

3.1 Food handlers specimens

3.1.1 Collection of specimens

A total of 1516 clinical specimens were collected from 428 foodhandlers of different nationalities, who apply to work in hospital-located kitchens in Makkah. These specimens represent 428 nasal swabs, 428 throat swabs, 428 nail swabs, 228 stool samples and 4 wounds swabs. Stool samples and wound swabs were taken if available. Food handlers were divided into two major groups; Arabs, and Asians to facilitate analysis and interpretation.

The Arab food handlers group included the worker from Saudi Arabia and Egypt While; the Asians were from Bangladesh, India, Indonesia, Nepal, Pakistan, Philippines, and Serilanka. Table17 shows that the total numbers of Arab foodhandlers were 45. This represent 11% of the total number of workers whereas, Asians were 383, which represent 89%. It was also observed that the total numbers of male food handlers were the majority amongst the total number of workers 428, also amongst the Arabs and Asians 40 (9.3%), 342 (79.9%) respectively. This may be due to traditional social customs. On the other hand, the proportions of the females amongst the Arabs and Asians were almost the same, i.e., 11.1%, 10.7 % respectively. The distribution of food handlers that were examined according to their nationalities and sex are shown in Fig.6.

Table (17): Distribution of examined foodhandlers at selected Makkah's hospitals.

Nationality	Food handlers		Males		Females	
	Total	Percentage	Total	Percentage	Total	Percentage
Arabs	45	10.5%	40	9.35%	5	1.17 %
Asians	383	89.5 %	342	79.9%	41	9.6 5%
Total	428	100%	382	89.25%	46	10.75 %

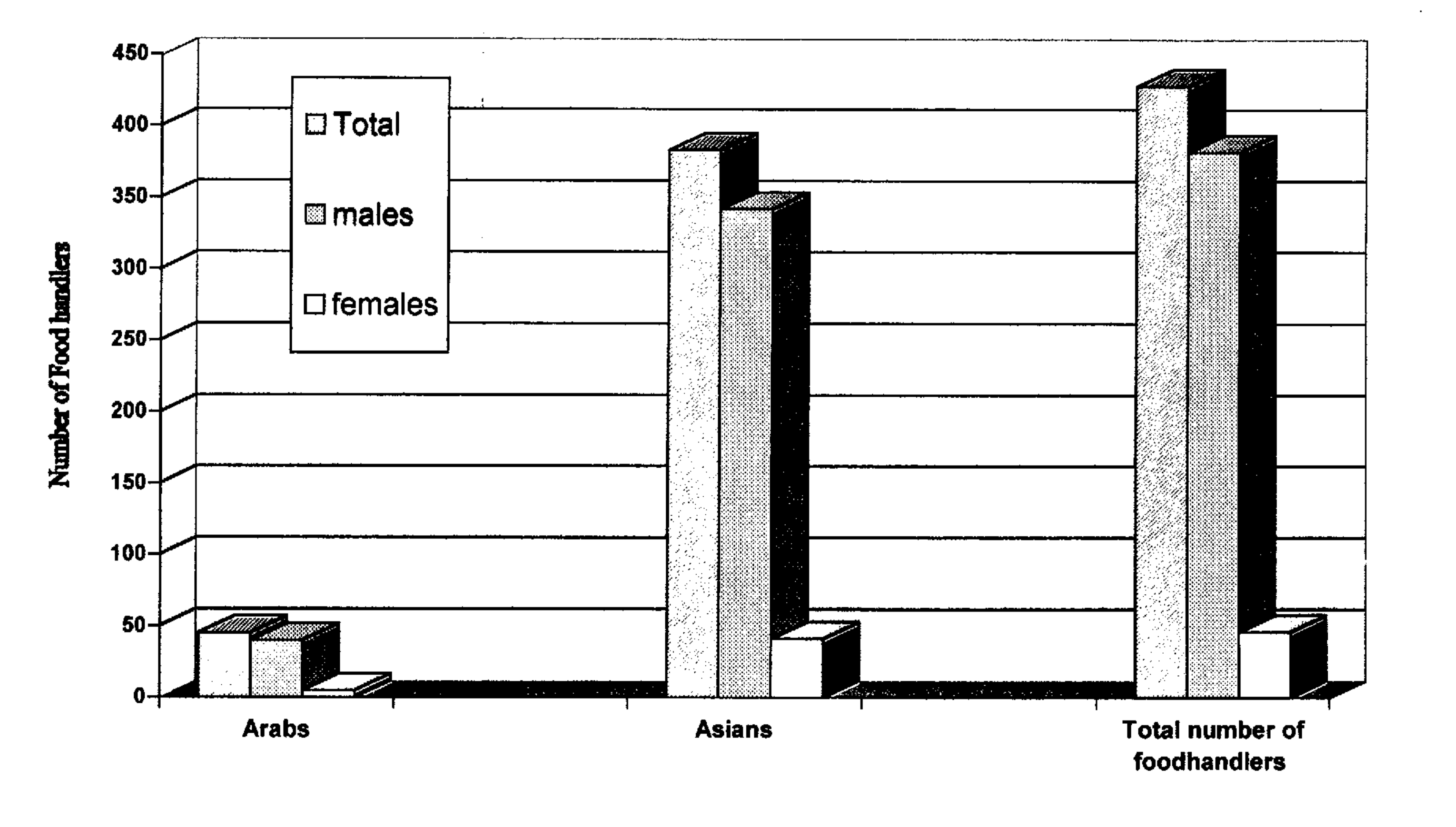


Fig. 6: Distribution of Food handlers examined according to their nationalities and sex.

3.1.2 Isolation and characterization of *S. aureus*

All specimens were sub-cultured directly on blood agar and mannitol salt agar plates. These were incubated for twenty-four hours at 37°C. However, the temperature of incubation was monitored using a 7days recording chart thermometer. Fig.7 showed that the temperature was stable all time. Colonies observed on Blood agar (Fig.8), Mannitol salt agar (Fig.9), and Baird Parker agar (Fig.10) were morphologically investigated. Any 1.5-2.5mm round, smooth, raised, glistering colonies and usually opaque producing pigments that vary from white to cream, on the Blood agar, or yellow to orange on Mannitol salt agar were designated as *S.aureus*. Nevertheless, all colonies appear to be *S.aureus* were tested for coagulase (see section 2.3.2.) were showed in Fig.11.

On the other hand, any sign of haemolysis is regarded as positive result because the term α, β, γ, and δ haemolysis should not be used with genus *Staphylococcus* (Devriese and Hajek, 1980). Also the use of the mannitol and the acid production, which change the color of the Mannitol salt agar media to yellow, confirmed that colonies are belonging to *Staphylococcus aureus* (Fig.9). The colonies confirmed to be coagulase positive were subjected to further tests such as; Catalase test (Fig.13), Gram staining, DNase test (Fig.12), and Oxidase test (Fig.14).

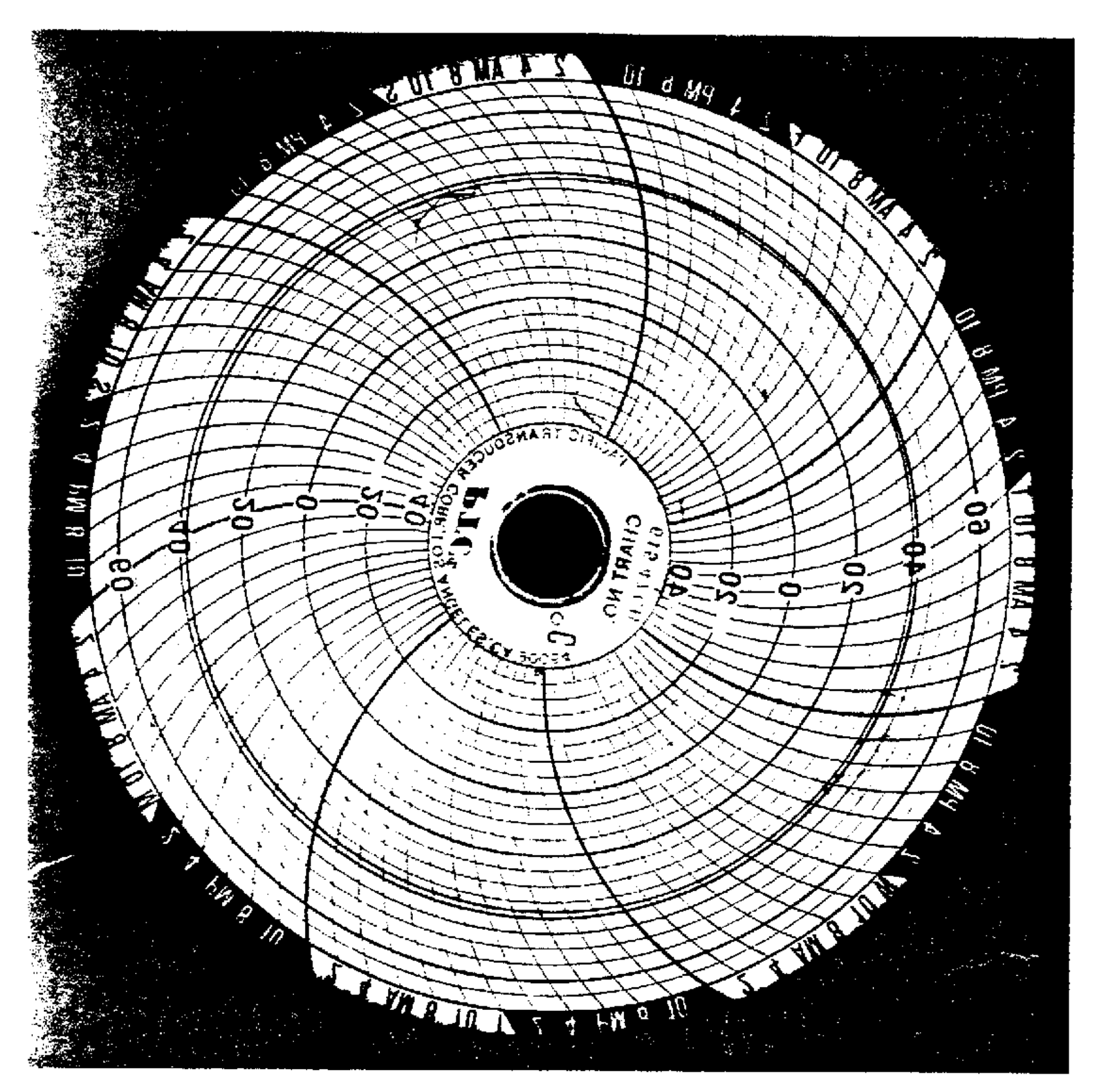


Fig. 7: Chart recorder of incubator temperature during 7days



Fig.8 Typical colonies of *S.aureus* on Blood agar plates

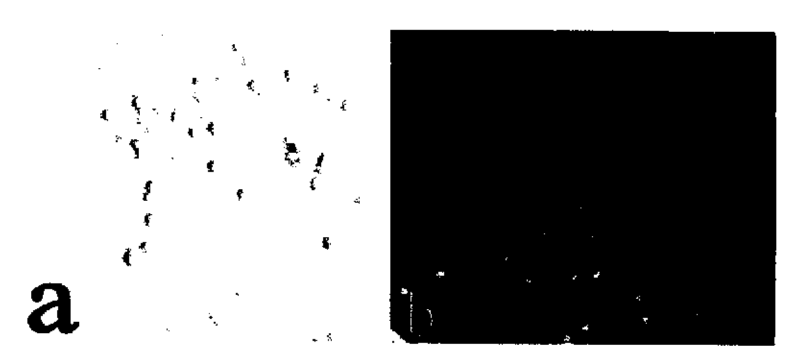


Fig.9: *S. aureus* on Mannitol Salt agar a: +ve and b: -ve reaction



Fig.10: Typical colonies of *S.aureus* on modified Baird Parker

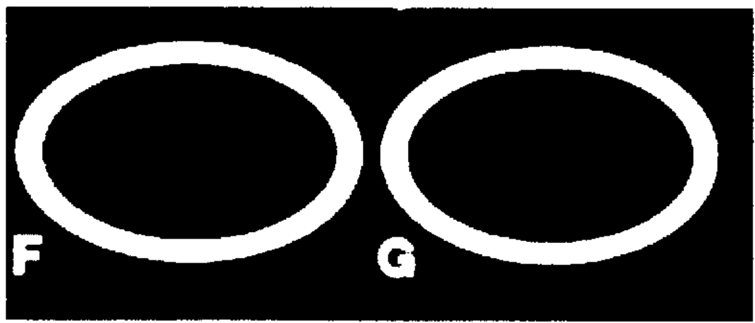


Fig.11: Coagulase test F: positive reaction, G: negative reaction

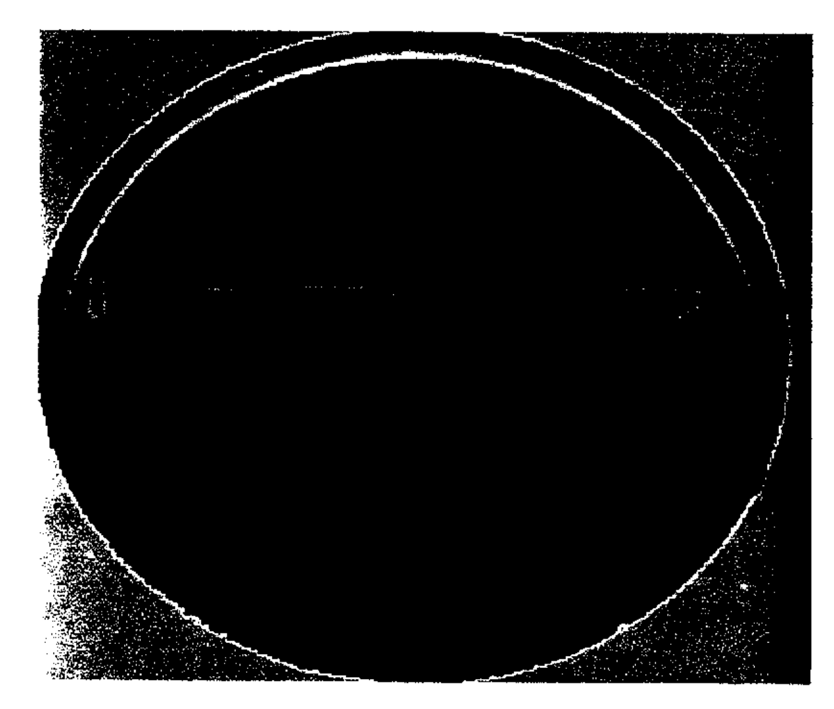


Fig.12: DNase Test: a positive and b: negative results.

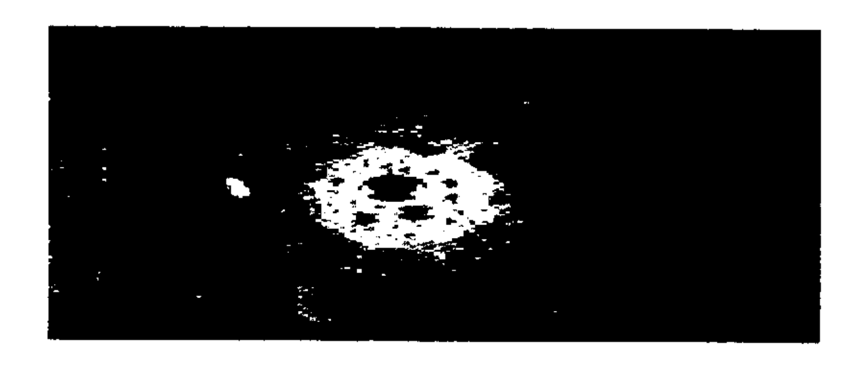


Fig.13: Positive reaction of Catalase test



Fig.14: Oxidase test a: positive and b: negative reaction

Of 1516 clinical specimens, 129 *Staphylococcus aureus* isolates were catalase positive, coagulase positive, DNase positive. These isolates belong to thirty-eight Arabs and Asians food handlers. This is in agreement with those obtained by Soares *et al* (1997) who reported that from 10-50 % of the human populations are healthy carriers of *S. aureus*. On the other hand, Al- Bustan *et al*., (1996) found that 26.6 % of 500 workers studied were screened for nasal carriage of *S.aureus*.

3.1.3 Enterotoxigenicity of the isolates

Of 129 isolates of *S. aureus* 45 (35 %) were found to produce enterotoxins A, B, C and D singly or in pairs, when evaluated by Reversed Passive Latex Agglutination test (RPLA). The SET-RPLA instructions were followed with slight modifications, i.e., incubation period for only overnight, which was sufficient according to the method of Wieneke (1991).

The incidence of enterotoxins A, B, C, and D production in isolates of *S. aureus* is presented in Table 18. Enterotoxins were shown to be produced by 35% of the isolates obtained from 29% of the total working food handlers who look healthy. These results are in agreement with those obtained by Hajek, (1978) who reported that 38% of strains were obtained from healthy persons, but Casman *et al* (1967) found that SEs produced by 50% of *S. aureus* strains isolated from clinical specimens, obtained from both

Table (18): Number of isolates producing enterotoxins according to source

Source of isolates	NO. of isolates producing enterotoxins	Enterotoxins produced according to source						
		A	B	C	D	A,B	A,C	D,C
Nasal swabs	29	11	1	11	1	2	2	1
Throat swabs	9	2	-	5	-	1	1	-
Nail swabs	6	3	-	3	-	-	-	-
Stool samples	1	-	-	1	-	-	-	-
Total	45	16	1	20	1	3	3	1

patients and healthy individuals. This figure of 35% may be on the lower side of the scale, as other enterotoxins SEE, SHE, SEG and SEI were omitted from the study because simple detection method was not readily available. Also coagulase negative staphylococci were not subjected to enterotoxin evaluation, and they may be a potential cause of food poisoning (Udo *et al.*, 1999).

Thirty-eight isolates (29.5%) produced only one enterotoxin; of these 20-produced C (15.5 %), 16 strains produced enterotoxin A (12.4%), one produced B (0.7%), and one produced enterotoxin D (0.7%). Isolates that produced one enterotoxin alone were most common, which agreed with the findings of Casman *et al.*, (1967), Payne and Wood, (1974), Roder *et al.*, (1995), and Soares *et al.*, (1997). However, seven isolates (5.4%) produced more than one enterotoxin; 3 isolates produced A and B (2.3%), 3 isolates produced A with C and only one isolate produced C and D.

The predominance of specific enterotoxin types among *S.aureus* isolates from human carriers is variable. Casman *et al.*, (1967) found that SEA and SED occurred most commonly in the strains of S. *aureus* isolated from clinical specimens. In contrast, Reali (1982) found that *S. aureus* strains producing SEB were the most common isolates. Whereas, Melconian *et al.*, (1983) found that 27.7 % of the isolated *S. aureus* produced SEA, 15.3 % produced SEB and 6.2% produced SEC. Similarly, Al-Bustan *et al.*, (1996) found that 28 % produced SEA, 28.5 % produced SEB, 16.4 % produced SEC, and 3.5 % produced SED. While Adesiyun *et al.*, (1986) found that SEA was produced by 32.7% of all

enterotoxigenic strains, while SEC and SED were produced by only 6.8% and 6.3% respectively, of the strains examined. On the contrary Roder *et al.*, (1995) found that enterotoxin B and C were the toxins produced most frequently in both groups, which were isolated from blood cultures of suspected cases and those from healthy carriers. Quite similarly, Soares *et al.*, (1997) found that 50% of the *S. aureus* isolates produced SEC, 23.1 % SEA and 15.4% SEB. The present study, which is the only study until now in Saudi Arabi, found that 15.5 % of the *S. aureus* produced SEC and 12.4 % SEA. So that they were the enterotoxins produced most frequently

A predominance of SEC and SEA- producing *S. aureus* strains among food handlers has been reported by Melconian *et al.*, (1983), Marin *et al.*, (1992), Roder *et al.*, (1995), and Soares *et al.*, (1997) In 1978, Hajek suggested that the source of these enterotoxin C producing strains is unclear and have related enterotoxin C to strains of animal origin. However, the interchange of the staphylococci between animals and man might explain the higher incidence of SEC.

Enterotoxigenicity of *S. aureus* isolates according to the source of the specimen's collection were summarized in Fig.15. Most of enterotoxigenic *S. aureus* were isolated from nose. Nevertheless, twenty-nine strains of total food handlers' harbored enterotoxigenic

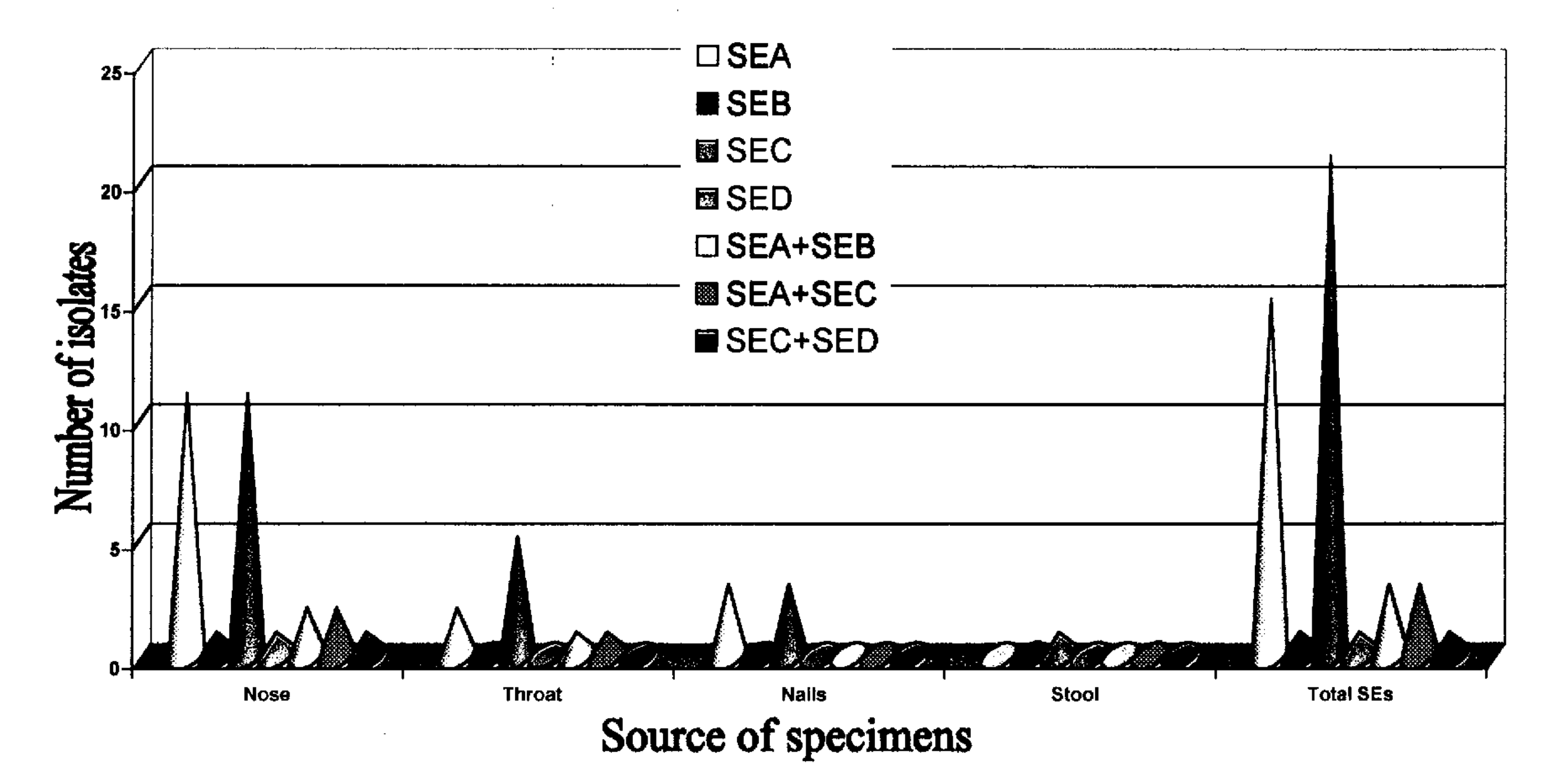


Fig. 15: Enterotoxigenicity of *S. aureus* isolates according to the source of specimens

Table (19): Carriers food handlers

Nationality	Number of Carriers			Total number of food handlers			Total Percentage
	Females	Males	Total	Females	Males	Total	
Arabs	1	3	4	5	40	45	8.8%
Asians	5	29	34	41	342	383	8.9 %
Total	6	32	38	46	382	428	8.9 %

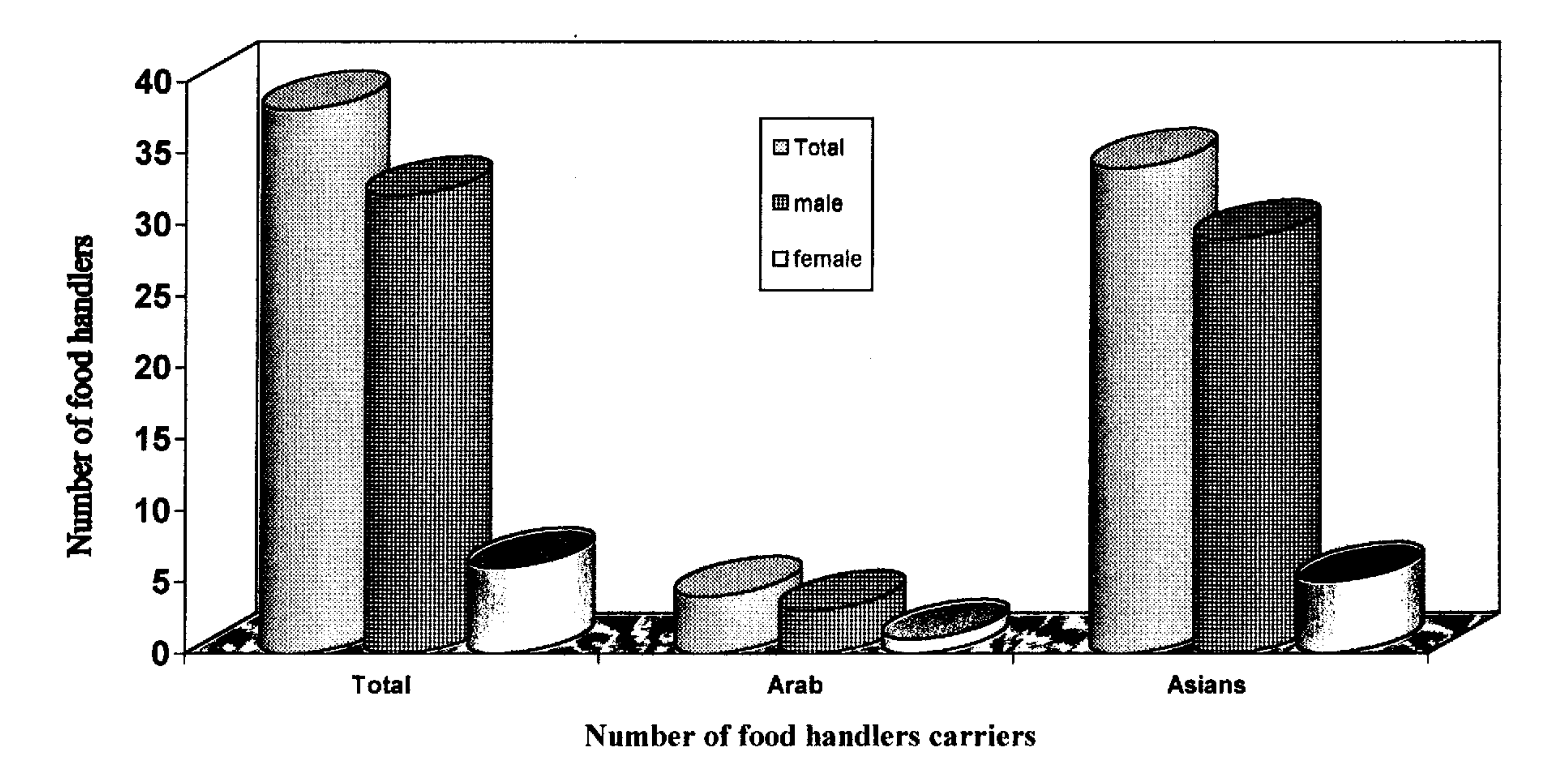


Fig. 16: Carriers food handlers

S. aureus in their anterior naris while nine strains were isolated from throat. Only six isolates were observed in nail specimens. This phenomenon was also reported by Soares *et al.*, (1997). He referred this to implementation of hospital control measures to stop transmission of epidemic bacteria. In this study, it might be related to catering companies' instructions to the food handlers. Because they have signed a contract to operate the kitchens of the hospitals, and to employ healthy food handlers whom have health certificates. Table 18 shows the total number of foodhandlers that subjected in this study and staphylococcal carriers. The total ratio of both Arabs and Asians carriers were quite the same (8.8 and 8.9 respectively). Also the percentage of both males and females were 8.4 and 13% respectively.

Bad habits such as picking nose (fingering the nose), nasal secretions and spiting on the ground increase the ratio of staphylococcal contamination of foods. Also mishandling and disregarding hygienic standards particularly during Hajj seasons when food handlers become under pressure to prepare large quantities of food. In addition, lack of experience and poor hygienic practices cause food contamination. On the other hand, some customers may save their cooked food to be consumed after several hours under unsuitable conditions allowing microbial population particularly staphylococci to multiply. Such delay in consumption without protection will permit enterotoxin production in food. Detection of enterotoxigenic *S. aureus* in cooked food and food handlers is very important in the cases of food poisoning. Because

their presence does not necessarily imply that enterotoxin was produced. Vice versa, the absence of viable staphylococci in food does not mean that toxins are not present.

Humphreys *et al.*, (1989) Roder *et al.*, (1995), Soares *et al.*, (1997), and Krakauer, (1999) suggested that the production of the enterotoxins by *S. aureus* isolates may also be important in the pathogenesis of infections rather than staphylococcal food poisoning.

Two *S.aureus* isolates that produced SEA and SEC respectively were subjected to further studies such as measuring growth curve in liquid medium and Saudi traditional cooked rice. Also, the determination of minimum detectable amounts of SEs in cooked rice using RPLA, and the study of the effect of different holding temperatures on the production of staphylococcal enterotoxins in Saudi traditional cooked rice were determine later.

3.1.4 Sensitivity test

To observe the differences between these isolates and their susceptibility to some antibiotics, the sensitivity test was performed. The results of the Antimicrobial sensitivity test for 45 *S. aureus* isolates producing enterotoxins are presented in Table 20. The isolates showed different antibiotic resistance patterns (as exampled in Fig. 17). The isolates fall into 7 groups according to their reaction to the antibiotics, so that each group has the same antibiotic resistance pattern. Of the eight Antimicrobial agents used in Kirby-

Table (20): *S. aureus* isolates classified according to sensitivity reactions

S.aureus isolates with same sensitivity (Numbered)	Type of SEs	Antibiotic							
		cephalothin	Clindamycin	Oxacillin	Cotrimoxazole	Erythromycin	Gentamicin	Oxytetracycline	Penicillin
Group (1)									
No. 1- 11	C	S	S	S	S	S	S	S	R
12-25	A	S	S	S	S	S	S	S	R
26-28	AB	S	S	S	S	S	S	S	R
29-30	AC	S	S	S	S	S	S	S	R
31	D	S	S	S	S	S	S	S	R
32	DC	S	S	S	S	S	S	S	R
Total: 32 isolates									
Group (2)									
No. 33-37	C	S	S	S	R	S	S	S	R
38	AC	S	S	S	R	S	S	S	R
39	B	S	S	S	R	S	S	S	R
Total: 7 isolates									
Group (3) No.40	C	R	S	S	R	S	S	S	S
Group (4) No.41	A	S	S	S	S	R	S	S	R
Group (5) No.42	A	S	S	S	S	S	S	R	R
43	C	S	S	S	S	S	S	R	R
Group (6) No.44	A	R	S	S	S	S	S	S	R
Group (7) No.45	C	S	S	S	R	S	S	R	R

S: sensitive R: resistant

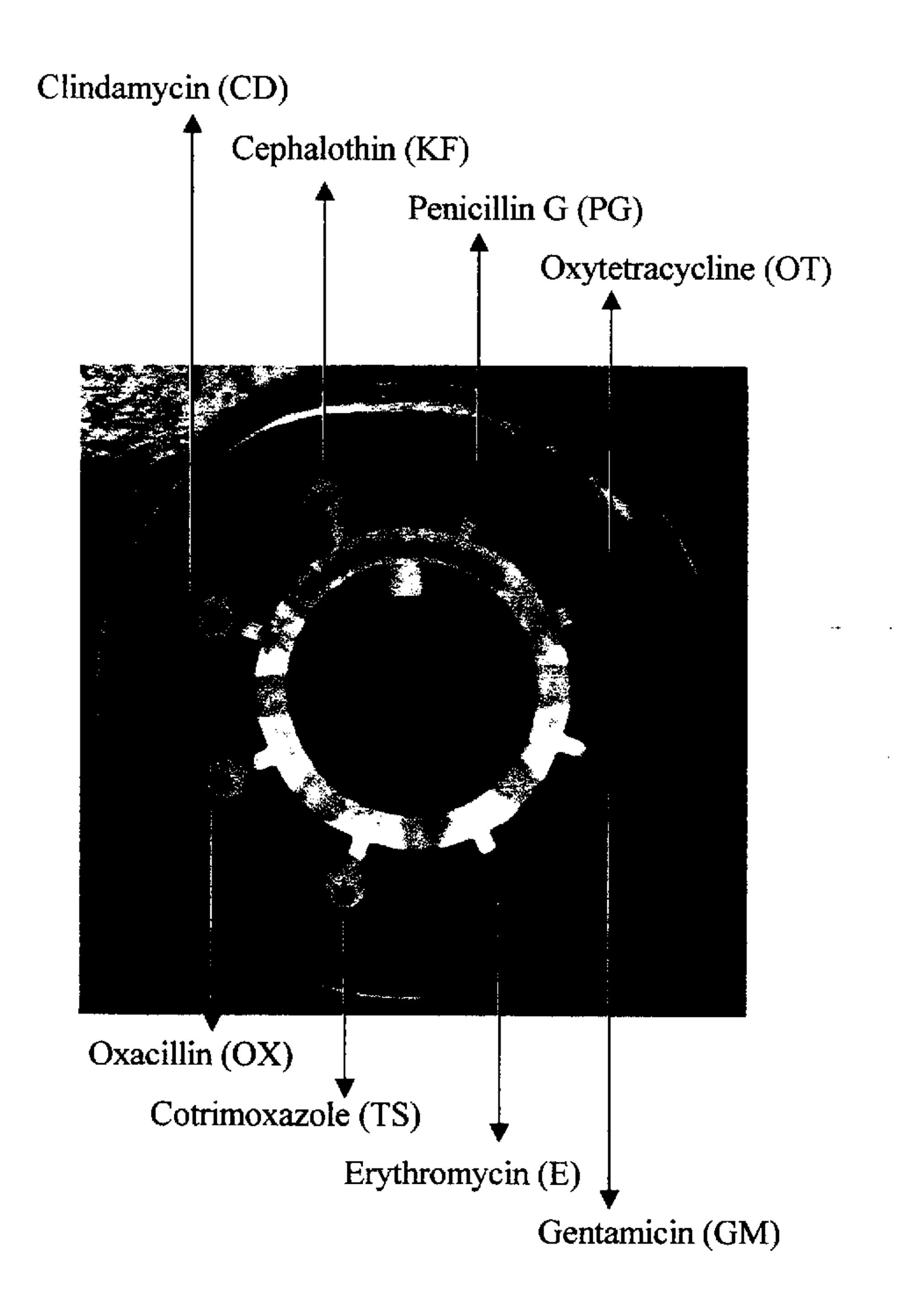


Fig.17: Example of sensitivity test for *S. aureus* using various antibiotics

Bauer method, *S.aureus* enterotoxins producers' isolates were most resistant to Penicillin G (97.8%). This was similar to the results (94 %), which reported by Soares *et al.*, (1997). This might be related to the frequent administration of the drug. On the other hand, almost all *S. aureus* enterotoxins producers' isolates were sensitive to Clindamycin, Gentamicin, and Oxacillin. Within this group, 93.3 % of the isolates were sensitive to Oxytetracycline (OT), whereas95.5 % of them were sensitive to Cephalothin (KF). Increased resistant (20 %) to Cotrimoxazole (TS) was found in the nasal isolates.

An interesting observation is that some isolates from different locations of the body of three food handlers exhibited the same antibiotic resistance patterns (Nasal, Throat, Stool) (Nasal, Throat, Nails) (Nasal, Nails), type C, A, and A respectively. It could be noticed that there are cycles of contamination and cross contamination between these organs. On the other hand, three different isolates from different locations (Nasal, Throat, Nails) of one female food handler had different antibiotic resistance patterns. Which indicate the susceptibility of one person to harbor or be infected with different strains of *S. aureus*.

3.1 Collection of Mandy rice samples

Twenty-four rice samples of Mandy were obtained from six different restaurants in Makkah. Restaurants were chosen randomly and designated as R1, R2, R3, R4, R5, and R6. . Samples were collected from pots at noon, when the restaurants begin to operate, and ten hours later for two days.

Table (21): Average temperature of rice samples in pots

Restaurant / Time	R1	R2	R3	R4	R5	R6
12:00 pm	59.5°C	78.5°C	73.5°C	59.8°C	66°C	64°C
10:00 pm	56.5°C	56°C	53°C	53°C	54.5°C	47°C

3.2.1 Determination of variability in cooked rice temperature

Determination of variability in rice temperature of specimens during ten hours of incubation in pots were summarized in Table 21.The range of rice temperatures at noon varied between 59.5 to 78.8 °C. However, it was between 47 and 56.5°C during night. The range of rice temperature drops from twelve to twenty two degrees during night in most restaurants. Nevertheless, R1 and R2 exhibit better results compared with others. The range of rice temperature drops in both restaurants from three to seven degrees only. As shown above, sanitation in most of those restaurants are in compliance with the regulations of WHO. Variation in rice temperatures allows bacterial growth particularly at night (see section 3.2.2). This is one of the most factors contributing to outbreak of staphylococcal food poisoning (Hobbs and Roberts, 1990).

Two samples from restaurants R1 and R4 respectively, which have been taken at 12:00 am had relatively low temperatures; this could be due to inadequate preheating or reheating of left over rice. Because most of the fresh cooked rice temperatures were higher than 60 °C even if more than one hour has already been passed after cooking.

3.2.2 Bacterial total count

Standard plate counts were made for samples that were taken from the large cooking pots when opened soon after cooking (12:00 am) and also after ten hours of serving (10:00 pm). This can

demonstrate how effective is the holding conditions on increasing the contaminating bacterial numbers.

From the results presented in Table 22 it appears that sanitary qualities were best at noon, when the pots were not opened too much and the temperatures were relatively high. However, it may indicate insanitary handling, or unfavorable holding temperatures the same in samples 4,and 13, which have been taken at 12:00 am. On the other hand, it is necessary to avoid the wrong interpretation of low plate counts, since it is possible for the cupidity cooker to preheat or reheat the Left-over by addition of hot gravy or sauce which can kill the vegetative cell but not destroy the enterotoxins, and this could be noticed in Restaurant 1and 4 in samples 4,and 13, because the samples have been taken at 12:00 am. However, the relation of the Left- over food and incidences has been reported in many cases as been reported by Hobbs and Roberts (1990).

However, 73.8 % of the total samples showed no growth, and 29.2 % contain between 2-10 $\times 10^5$ cell/gm. This is lower than the results obtained by Jaad, (1997) who found that 67% of samples were rejected because they contain more than 0.1×10^6 cell/gm, and higher than the results obtained by Ghazoli, (1994) who reported that 2.3% of samples were rejected. The type of the rice and the difference of total number and various kinds of dishes presented by restaurants could explain the variation of the results. The restaurant that sells only one type of food may concentrate more than that

Table22. The Bacterial total count (CFU/gm) of Mandy rice samples

Restaurant	Sampling at	Rice Temp.	CFU at 37°C	CFU at 55°C
R1	12pm	52°C	30	0
R1	10pm	57°C	Nil	Nil
R1	12pm	67°C	Nil	Nil
R1	10pm	56°C	Nil	Nil
R2	12pm	82°C	Nil	Nil
R2	10pm	55°C	Nil	Nil
R2	12pm	75°C	Nil	Nil
R2	10pm	57°C	Nil	Nil
R3	12pm	78°C	Nil	Nil
R3	10pm	51°C	10	2000
R3	12pm	69°C	Nil	Nil
R3	10pm	55°C	Nil	Nil
R4	12am	50°C	20	4000
R4	10pm	50°C	10	0
R4	12pm	67°C	Nil	Nil
R4	10pm	56°C	Nil	Nil
R5	10pm	65°C	Nil	Nil
R5	10pm	52°C	10	0
R5	12pm	67°C	Nil	Nil
R5	10pm	57°C	Nil	Nil
R6	12pm	59°C	Nil	Nil
R6	10pm	45°C	200000	15000
R6	12pm	69°C	Nil	Nil
R6	10pm	49°C	600	10000

presents different kinds of dishes in many respects relating to efforts concerning preparation and sanitation

3.2.3 pH of The rice samples

Aside from temperature, the hydrogen ion concentration of the rice exerts a limited influence on the growth of *S. aureus* since it can grow in a wide rang of pH (Genigeorgis and Sadler, 1966). However, the average of the pH of the rice samples were: 4.7 for Bokhary rice and for Mandy rice is 5.5. It seems from the results that the average of Bokhary rice was relatively lower than the average of Mandy rice.

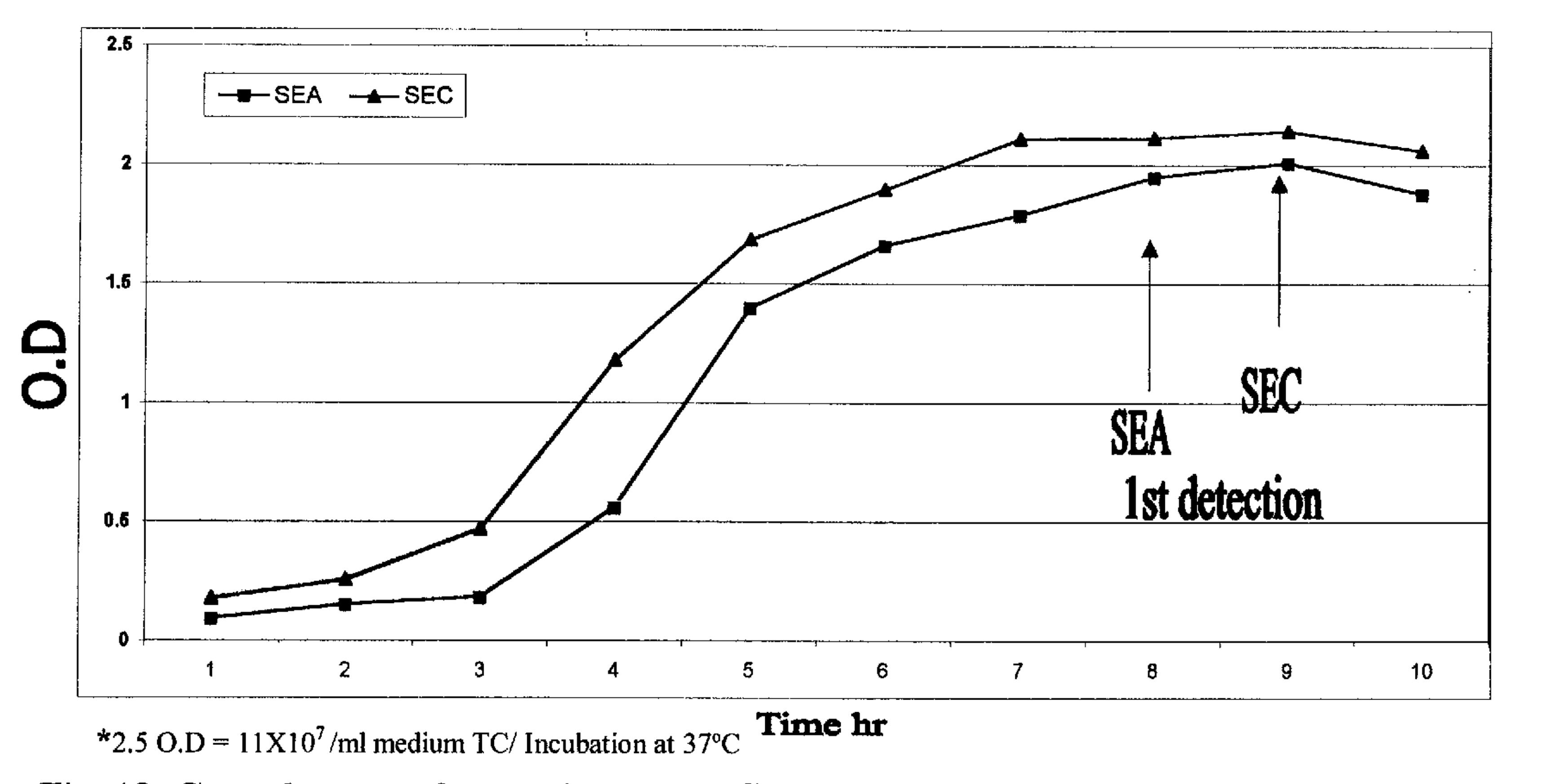
3.2 *S. aureus* enterotoxigenicity studies on rice

3.3.2 The selected cultures

Enterotoxins C and A, showed the highest percentage in the foodhandlers specimens. So, two isolates each forming only one enterotoxin were chosen. They have been subjected to further studies: such as growth cycle in the medium and in the cooked rice, the effect of the holding temperature on the production of SEA and SEC

3.3. 2 Growth curve of *S. aureus* in synthetic medium

The growth curves of the chosen two isolates, which produced either SEA or SEC, are shown in Fig 18. The generation time for SEA producing isolate was 37.1 min. Enterotoxin A was first



*2.5 O.D = $11X10^7$ /ml medium TC/ Incubation at 37°C

Fig. 18: Growth curves for two isolates of *S. aureus*, which produce SEA and SEC in (BHI)

detected after approximately 8 hours of incubation. On the other hand, the generation time for the SEC producing isolate was 30min. Enterotoxin C was first detected after 9 hours of incubation. In both isolates the concentration of the enterotoxin increased as the growth curve progresses.

3.3. 3 Growth curves of *S. aureus* in rice dishes

The growth curves of *S. aureus* in Mandy and Bokhary rice are shown in Fig.19 and 20 respectively. The organism grew in both kinds of rice at 45°C. The population of the examined *S. aureus* in Mandy rice increased from 1.3×10^{6} to $1.4\text{x}10^{10}$, whereas the population increased in Bokhary rice from 2.6×10^{6} to 2.4×10^{9} within 24 hrs. It could be noticed that the growth was definitely faster in Mandy rice than in Bokhary rice. The difference could be attributed to:

1. The pH of the Mandy rice dish was 5.5 while Bokhary rice was 4.7 due to the addition of tomato past and lemon juice, which is in agreement with Genigeorgis and Sadler (1966) who found that aerobic growth was prevented when the initial pH4.8 in the medium.
2. Spices content of Bokhary rice, cause inhibitory or interfering effect with the growth of *S. aureus* and other microorganism.

From the results it seems that SEA and SEC were produced at the beginning of the stationary phase. However, Otero *et al.*, (1990)

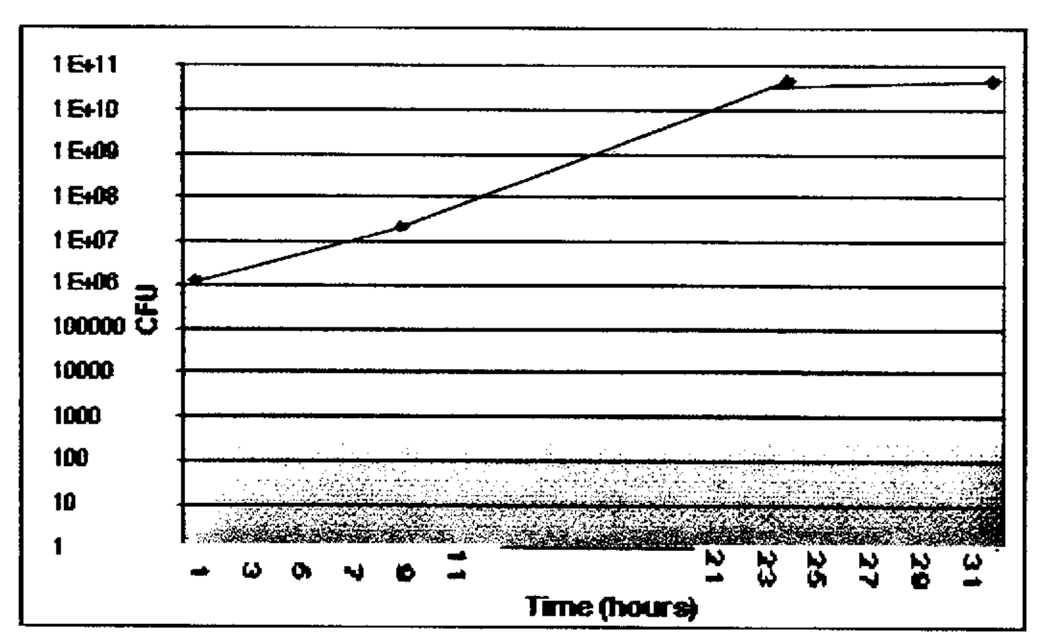


Fig.19: Growth curve for *S.aureus* in Mandy rice

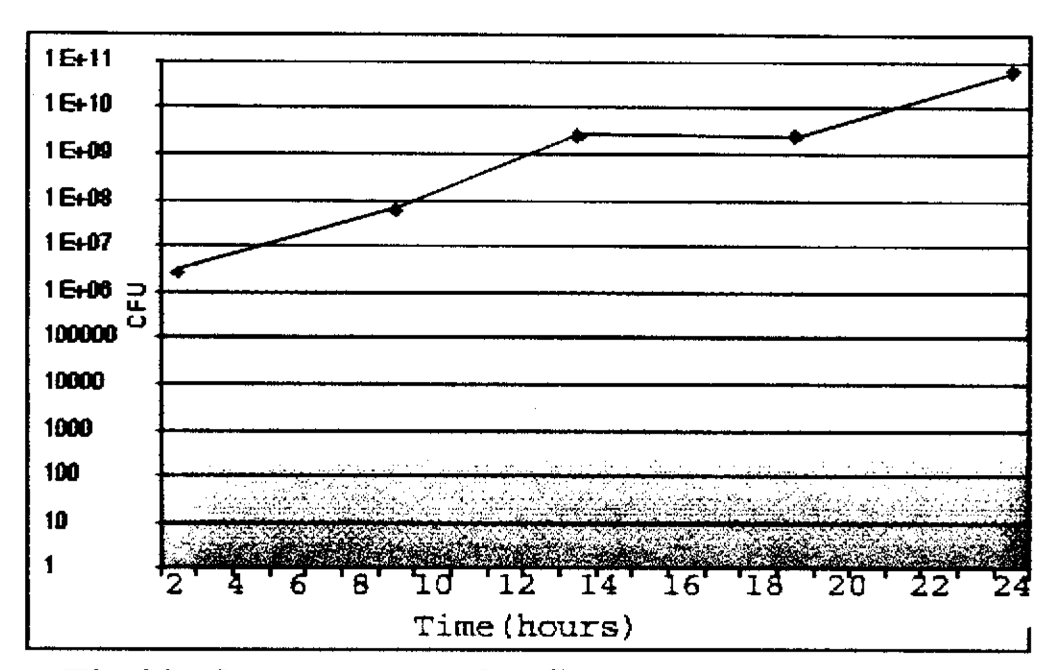


Fig.20: Growth curve for *S.aureus* in Bokhary rice

indicated that the enterotoxin C starts to appear at the late logarithmic phase in some strains while in the beginning of the stationary phase in other. Which is in agreement with our findings with SEC. However, the minimum incubation period and the the lowest population of *S. aureus* associated with detectable enterotoxin are dependent on the strain and was expressed as toxin yields after 24h. On the other hand, these results succeeded in demonstrating that enterotoxins are usually produced in detectable amounts after the count of staphylococci reaches a population of about 10^6/g.

3.3. 4 Minimum detectable amounts of SEs in rice by RPLA

Several trials have been done by the addition of various amounts of the reconstituted enterotoxin (the supplied lyophilized enterotoxin vial contain 100ng/ml after the reconstitution with 0.5 ml diluent) to 10g of cooked rice. After the centrifugation and for maximum enterotoxin recovery the method ascribed in the kit's manufacture was followed. However, this procedure of Denka Seiken Co. did not work, so the method of Park and Szabo (1986) was used. The latter method was found very sensitive to detecting the enterotoxins in the Mandy and Bokhary rice. The main difference between both methods is the use of the refrigerator centrifuge, which accumulates the oil at the top of the tube. So, the supernatant will be free from the oil. 3ml syringe was used to withdraw the supernatant from underneath the oily layer.

Table (23): The minimum detectable SEs in rice by RPLA

Enterotoxin	Dilutions								
	1/10	1/20	1/40	1/80	1/90	1/100	1/120	1/140	1/200
A	+3	+3	+3	+2	+	+/-	+/-	-	-
B	+3	+3	+3	+2	+	+/-	+/-	-	-
C	+3	+3	+3	+	+	+/-	-	-	-
D	+3	+3	+3	+	+	+/-	-	-	-

In this study, an initial dilution of 1:10 was used followed by serial dilations, i.e., 20,30, 40, 80, 90, 100,120, 140, and 200. The agglutination reactions were positive with the homologous antibody coated latex until 1:100 for SEC and SED. However, for SEA and SEB the detection reached 1:120. The results are presented in Table 23. These were in agreement with the results that reported by Wieneke (1988), who found that strong agglutination reactions were still obtained with 1:100 dilutions.

Since enterotoxin C has three forms (Munson *et al.*, 1998) a cross-reaction can occur between different forms of the enterotoxin C, which was not differentiated in the present investigation. Therefore, the findings are in agreement with those reported by Wieneke (1988) and earlier by Reiser *et al.*, (1984). This means that RPLA kit is efficient in detecting six antigenically distinct enterotoxins SEA, SEB, SEC_1, SEC_2, SEC_3, and SED.

3.3. 5 The effect of rice temperature on staphylococcal enterotoxin production

3.3.5.1 Cooked rice

One isolates of *S. aureus* producing enterotoxin A and another one producing enterotoxin C (each isolates produce only one enterotoxin) were examined for their ability to grow and produce their toxins in Mandy and Bokhary rice dishes. The samples of rice were incubated for 3days. The enterotoxins were not detected throughout the incubation period. This could be attributed to the presence of contaminating microorganisms during

the handling of the rice and may have an effect on enterotoxin production during this extended period. Therefore, attempts were made to eliminate their effect by sterilizing the samples of cooked rice before inoculation.

EL-Nockrashy (1985) demonstrated that *S. aureus* inhibition might occur as a result of production of hydrogen peroxide by lactic acid bacteria. However, Genigeorgis *et al.*, (1971) reported the loss of viability of *S. aureus* in acidified media. The data presented show that enterotoxin cannot be present in foods when a non-enterotoxigenic *S. aureus* is greater than an enterotoxigenic strain.

3.3.5.2 Cooked then sterilized rice

For this experiment the rice samples were autoclaved at 121°C for 15 min. Then the prepared cell suspension of *S. aureus* was inoculated in the autoclaved rice. At first the inoculated rice samples were incubated for three days followed by testing for the presence of the enterotoxin. If the test was positive the experiment was repeated an incubation period of 48 hrs followed by experimenting for 24 hrs. Later, the incubation period was tested for presence of the enterotoxin every 6 hrs, and finally every one hour.

- Mandy rice

From the results presented in Fig. 21 it seems that SEA was detected after 16 hours in Mandy rice when the incubation temperature was 23°C, and was first detected after 6 hours when the incubation's temperature was 45°C. whereas the SEC was detected after 18 hours when the incubation temperature was 23°C, and after 6 hr at 45°C.

- Bokhary rice

From the results presented in Fig.22 it seems that SEA was detected after 10 hours in Bokhary rice when the incubation temperature was 23°C, and was detected after 6 hours when the incubation temperature was 45°C. while the SEC was detected after 11 hours when the incubation's temperature was 23°C, and after 6hr when the incubation temperature was at 45°C.

In both cases no significant effect of pH changes in Bokhary and Mandy rice during incubation. And this seems logic because most people can't easily differentiate between wholesome food and that containing enterotoxin from *S. aureus*. Also from the results it appeared that the production of enterotoxin A was faster than that of enterotoxin C, which is in agreement with the results of Shinagawa *et al.*, (1982), who found that SEA was produced more rapid than SEB and SEC. On the other hand, the production of SEA and SEC was more rapid in Bokhary rice than in Mandy rice, which could be attributed to the method of cooking and preparing

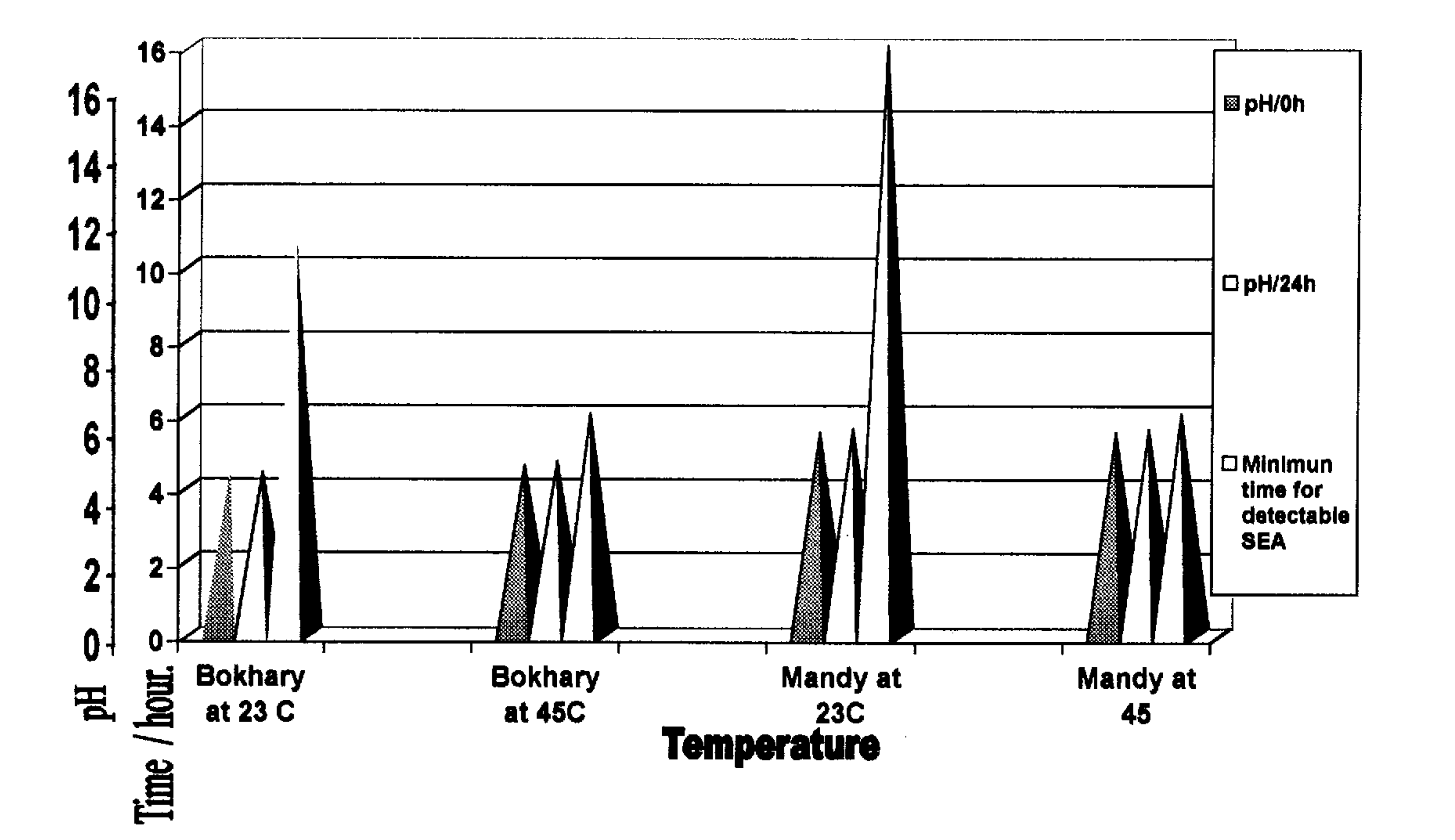


Fig.21: Effect of different temperatures on SEA production in Bokhary and Mandy Rice

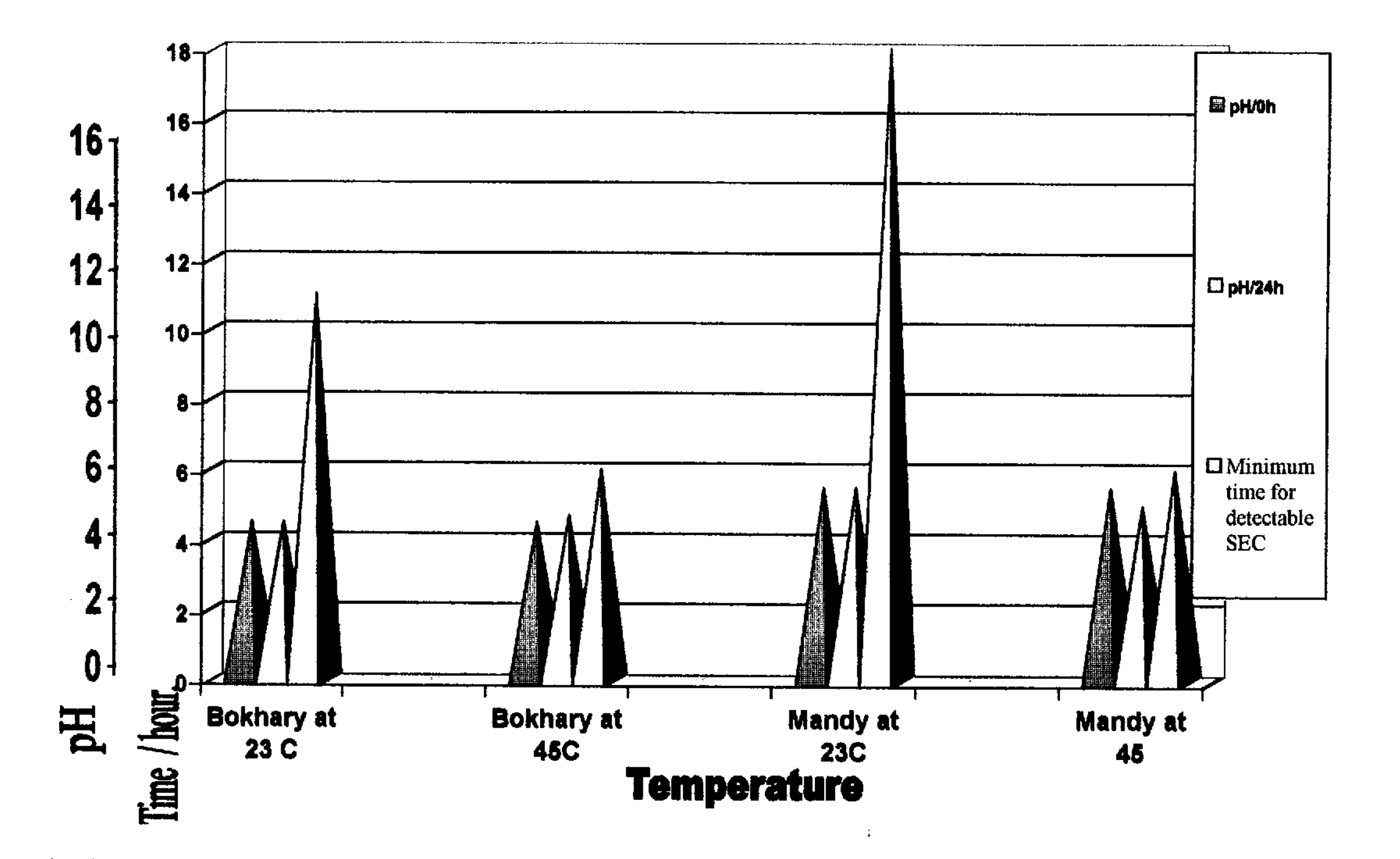


Fig.22: Effect of different temperatures on SEC production in Bokhary and Mandy Rice

Summary and Conclusion

Many inhabitants in Saudi Arabia nowadays depend on prepared cooked foods presented in many restaurants and pantries especially during Hajj and Omra seasons. In normal times, food handlers are subjected to medical examination before assignment to work in food stations. However, during high seasons of work, i.e., Hajj those establishments employ temporary workers; mostly lacking training in food handling operations, and sanitary practices, which are not easily enforced during excessive demand than these establishments can afford. This situation can encourage contamination with microorganisms both causing food spoilage and food poisoning. Of most widespread intoxication, which depends largely on sanitary practices is Staphylococcal food poisoning.

The present investigation is concerned with isolation, purification, and identification of *Staphylococcus aureus* isolates from some food handlers whom applied to work in hospital-located kitchens in Makkah during high seasons of Hajj. The isolates were characterized and tested for their ability to produce enterotoxins in culture media, and in some Saudi traditional cooked foods such as Mandy and Bokhary rice, which are very famous, in most Saudi dishes, and served in public kitchens spreading everywhere.

Out of 129 *Staphylococcus aureus* isolates from 1516 clinical specimens obtained from food handlers of different nationalities in Makkah; 35% produced enterotoxins A, B, C, and D singly or in pairs, when such enterotoxins were evaluated by Reversed Passive

Latex Agglutination test (RPLA). Most of the isolates are resistant to Penicillin G. However, they were sensitive to Clindamycin, Oxacillin and Gentamicin when tested by the Kirby-Bauer method.

Enterotoxins C and A, elaborated by 15.5% and 12.4%, isolates respectively, showed the highest percentage obtained. They were mostly isolated from nasal swabs rather than throat swabs. So, two isolates each forming either SEA or SEC were chosen, and were subjected to further studies, i.e., grow cycle in selected medium and in cooked rice. The effect of the holding temperature of rice on the production of SEA and SEC was evaluated.

On the other hand, 24 rice samples were obtained from six different restaurants in Makkah. The samples were taken at noon when the restaurants begin to operate and ten hours later for two days. The results of the samples, which were taken at 12:00am,are relatively higher than at 10:00 pm in respect to TC. This means that the holding temperatures became more suitable for the contaminating bacteria to grow by time. However, the averages of pH of the rice samples were: 4.7 for Bokhary rice and 5.5 for Mandy rice. It seems from the results that the average pH of Bokhary rice was relatively lower than that of Mandy rice.

To determine the effect of rice holding temperature on staphylococcal enterotoxin production many test have been done and include: the minimum detectable amount of SEs in rice, which was less than one µg by RPLA kit. Also the growth curves of *S. aureus* in medium, and in rice were determined. Finally, SEA and SEC were produced in Bokhary rice within less than twelve hours

when the holding temperature was 23°C and within less than seven hours when the holding temperature was 45°C. On the other hand, for the Mandy rice SEA and SEC were produced within less than 18 hours when the holding temperature was 23°C and within less than six hours when the holding temperature was 45°C.

Therefore, the following conclusion could be drawn:

1. The total ratio of both Arabs and Asians carriers were nearly the same (8.7 and 8.9 respectively). This indicates that bad habits such as; picking nose (fingering the nose), nasal secretions and spiting on the ground could be the reason for increasing the ratio of staphylococcal intoxication.

2. The predominance of specific enterotoxin type among *S. aureus* isolates from human carriers is variable.

3. Most of enterotoxigenic *S. aureus* were isolated from the nose.

4. The sensitivity results indicated the susceptibility of one person to harbor or be infected with more than one strain of *S. aureus*.

5. From the results of the rice holding temperature and bacterial total counts, it appears that sanitary at the start of

distribution of meals was better than at late hours; taking into consideration that the meal components are wholesome from the beginning.

6. The components of rice dishes and method of cooking have an effect on the production of *S. aureus* toxin in the food, as SEA was detected after 10 hrs in Bokhary rice and after 16 hrs in Mandy rice when the temperature of holding was 23°C while, the enterotoxins were detected after hrs if the holding temperature was increased to 45°C in both dishes.

7. Generally, no significant effect of pH changes in Bokhary and Mandy rice dishes on the production of *S. aureus* enterotoxins. This may due to the tolerance of *S. aureus* to changes in pH values. Also, due to the characteristics of *S. aureus* enterotoxins, that make it always difficult to differentiate organoleptically between wholesome food and that containing enterotoxins from *S. aureus*.

However, it is very necessary to reactivate the role of the health certification, so it should cover the training and educational part beside the medical examinations.

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Arabic summary الملخص العربي

يعتمد العديد من الناس على الأطعمة المعدة في المطاعم بمختلف أنواعها طول العام ،خاصة في موسم الحج حيث تكتظ المشاعر المقدس بما يقارب مليوني زائر. و نظراً لاختلاف جنسيات الحجاج واختلاف متطلباتهم من الأغذية، فأن المطاعم تقوم بالاستعانة بالعمالة الوافدة التي تخضع لكشف طبي عند بدء العمل و مراقبة طبية خلال العام من قبل السلطات الصحية . أما في موسم الحج فيتم الاستعانة بالعديد من "العاملين المؤقتين" في هذا المجال والذين ينقصهم في معظم الأحيان التدريب الكافي والتثقيف الصحي وبالتالي يسهمون في زيادة تلوث الأطعمة بالميكروبات الممرضة. ومن أكثرها انتشارا**S. aureus** المسبب للتسمم الغذائي العنقودي . وقد تضمنت هذه الدراسة عزل وتنقية هذا الميكروب وذلك عن طريق أخذ مسحات مختلفة شملت ١٥١٦عينة طبية أخذت من ٤٢٨ عامل أغذية متقدمين للعمل في مطابخ بعض المستشفيات في مكة المكرمة وهم من جنسيات مختلفة تم تصنيفها إلى عمالة عربية و أسيوية. وبعد فحصها تم عزل ١٢٩ سلالة و قد اختبرت قدرة هذه العزلات لإفراز السموم المعوية في البيئات وذلك باستخدام طريقة Reversed Passive Latex Agglutination test (RPLA) و قد أظهرت ٤٥ عزلة القدرة على إفراز السموم حيث بلغ عدد العزلات المفرزة للسم A وC ٢١و ١٥ عزلة على التوالي و ثلاث مفرزة للسم DC, D و B عزلة واحدة من كل نوع أما عدد العزلات المفرزة للسم A+B وA+C فبلغت ٣ عزلات .وقد أظهرت النتائج أن أعداد الحاملين لهذا الميكروب٣٨ عامل. أيضا تم إجراء اختبار الحساسية لهذه العزلات لمعرفة مدى تشابه هذه العزلات وقد وزعت النتائج العزلات إلى سبع مجموعات حسب نتيجة اختبار الحساسية .أيضا تم اختبار عزلتين تفرز السمين الأكثر انتشارا بين العاملين وهماA و C في بعض الأكلات المطبوخة مثل الأرز المندي و البخاري. وذلك لدراسة تأثير درجة حرارة الحفظ عن طريق تحضين عينات من الأغذية عند درجات حرارة ٤٥°م و٢٣°م وتلقيح الغذاء المعقم بهذه البكتيريا. و في سبيل ذلك تم اختيار ستة مطاعم وأخذ عدة عينات وتم تقدير درجة الحرارة للأرز في تمام الساعة الثانية عشر ظهرا وبعد عشر ساعات بمعدل عينتين. وأظهرت النتائج أن درجات حرارة الأرز في الظهر أعلى من المساء. كذلك تم عمل عد كلي لمعرفة مدى التلوث. وكانت نتائج جميع عينات المأخوذة ظهرا لا تحتوي على أي نموات عند استخدام بيئة nutrient agar ما عدا عينتين. أما عينات الفترة المسائية فقد احتوت خمسة عينات منها على نمو بكتيري. أيضا تم تقدير درجة الأس الهيدروجيني لجميع عينات الأرز. وكان المتوسط للرز البخاري ٤,٧ والمندي ٥,٥. كذلك تم تقدير اقل كمية من السموم التي يمكن الكشف عنها في الأرز وذلك باستخدام سموم مجفدة حيث أمكن الكشف على أقل من واحد ميكروجرام/جرام من الأرز.كذلك تم تتبع إنتاج السم من خلال دراسة منحنى النمو للعزلات المنتقاة. وبهذه الطريقة أمكن تحديد فترة التحضين الكافية لإنتاج السموم في هذه الأنواع من الأرز وتراوحت بين٦ إلى ١٨ساعة. كذلك تم اقتراح بعض التوصيات. مثل تفعيل دور الشهادة الصحية لتشمل الكشف والتثقيف الصحي.

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